

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C07K 14/435, C12N 1/00, 1/15, 1/21, 5/10, 15/12, 15/63	A1	(11) International Publication Number: WO 00/34320 (43) International Publication Date: 15 June 2000 (15.06.00)
(21) International Application Number: PCT/US99/29393 (22) International Filing Date: 10 December 1999 (10.12.99) (30) Priority Data: 09/210,330 11 December 1998 (11.12.98) US Not furnished 9 December 1999 (09.12.99) US (71) Applicant: CLONTECH LABORATORIES, INC. [US/US]; 1020 East Meadow Drive, Palo Alto, CA 94303 (US). (72) Inventors: LUKYANOV, Sergey Anatolievich; ul. Golubinskaya 13/1-161, Moscow (RU). FRADKOV, Arcady Fedorovich; ul. Dnepropetrovskaya, 35/2-14, Moscow, 113570 (RU). LABAS, Yulii Aleksandrovich; ul. Generala Tyuleneva, 35-416, Moscow, 117465 (RU). MATZ, Mikhail Vladimirovich; ul. Teplii stan, 7/2-28, Moscow, 117465 (RU). FANG, Yu; 583 Enos Street, Fremont, CA 94539 (US). CHEN, Ying; 680 Garland Avenue, #6, Sunnyvale, CA 94086 (US). HU, Lanrong; Apartment #2, 1281 Ayala Drive, Sunnyvale, CA 94086 (US). DING, Li; 1352 Norman Drive, Sunnyvale, CA 94087 (US). (74) Agent: ADLER, Benjamin, A.; McGregor & Adler, 8011 Candle Lane, Houston, TX 77071 (US).		(81) Designated States: JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF (57) Abstract The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are cDNAs encoding the fluorescent proteins.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
USES THEREOF**

10

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This is a divisional application of U.S.S.N. 09/210,330 filed
on December 11, 1998.

Field of the Invention

This invention relates to the field of molecular biology.
More specifically, this invention relates to novel fluorescent proteins,
cDNAs encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for
marking a protein, cell, or organism of interest. Traditionally, a
protein of interest is purified, then covalently conjugated to a
fluorophore derivative. For *in vivo* studies, the protein-dye complex is
then inserted into cells of interest using micropipetting or a method of
reversible permeabilization. The dye attachment and insertion steps,
however, make the process laborious and difficult to control. An

alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing

the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in general. However, the stability of GFP makes it sub-optimal for
5 determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of
10 which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great
15 utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities
20 based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

25

SUMMARY OF THE INVENTION

The present invention is directed to DNA sequences encoding fluorescent proteins selected from the group consisting of:

(a) an isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) an isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code and that encodes a fluorescent protein. Preferably, the DNA is isolated from a non-bioluminescent organism from Class Anthozoa. More preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 61, and the fluorescent protein has the amino acid sequence shown in SEQ ID No. 56.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising the DNA and regulatory elements necessary for expression of the DNA in the cell. Preferably, the DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56.

In still another embodiment of the present invention, there is provided a host cell transfected with a vector of the present invention, such that the host cell expresses a fluorescent protein. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells and insect cells.

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) isolated DNA from an organism from Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which

encodes a fluorescent protein. Preferably, the protein has the amino acid sequence shown in SEQ ID No. 56.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of:

5 (a) an isolated DNA which encodes a fluorescent protein, wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of

10 (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the organism is from Sub-order Endomyaria. Even more preferably, the organism is from Family Actiniidae, Genus *Anemonia*. Even more

15 preferably, the organism is *Anemonia majano*. Most particularly, the present invention is drawn to a novel fluorescent protein from *Anemonia majano*, amFP486.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide

20 probe for identification of a DNA encoding a fluorescent protein by means of hybridization, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.

25 Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers). In the case of *Anemonia majano*, the first degenerate primer used was NGH (SEQ ID No. 4), and the second degenerate primer used was GNG(b) (SEQ ID No. 10).

Figure 2 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 3 shows transient expression of pCNFPMut32-N1 and pECFP-N1 in 293 cells, respectively. PCNFPMut32-N1 (Figure 3A) shows brighter fluorescent intensity and less photobleaching compared to pECFP-N1 (Figure 3B). pCNFPMut32-N1 is constructed by amplifying Mut32 DNA and then inserting the amplified product into EGFP-N1 backbone.

Figure 4 shows that fusion protein PKC- γ -CNFP translocated from cytosol to the plasma membrane when cells were treated with PMA (Phorbol 12-Myristate 13-Acetate). Figure 4A shows the result from control (without the treatment) and Figure 4B shows the result from PMA-treated cells.

Figure 5 shows functional analysis of destabilized amFP486. Figure 5A shows that expression of pCNFP-MODCdl in HEK 293 cells exhibited purple fluorescence (pseudocolor). However, the actual color should be cyan (control). Figure 5B shows that transient transfection of pCNFP-MODCdl demonstrates 50% decreased fluorescent intensity after 4-hour treatment with protein synthesis

inhibitor cycloheximide. pCNFP-MODCdl is constructed using Mut32 DNA clone.

5 **Figure 6** shows histogram of wildtype amFP486. The fluorescent intensity of the transfected cells was analyzed on FACS using FL1 (510/30) detecting channel. Five samples (A-E) were analyzed in parallel. Geo Mean = geometric mean for data points.

Figure 7 shows histogram of Mut15. Same method as in Figure 5 was used. Five samples (A-E) were analyzed in parallel.

10 **Figure 8** shows histogram of Mut32. Same method as in Figure 5 was used. Five samples (A-E) were analyzed in parallel.

Figure 9 shows the expression of fusion protein Mut15-mdm2 in HEK293 cells.

Figure 10 shows the spectrum of wildtype amFP486. EX = 458 nm, EM = 492 nm, both slits = 2.5 nm.

15 **Figure 11** shows the spectrum of Mut15.

Figure 12 shows the spectrum of Mut32.

Figure 13 shows the spectra of wildtype and mutant amFP486 on the same graph.

20

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art
25 versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al.,

Nature 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

5 As used herein, the term "CFP" refers to cyan fluorescent protein, and the term "ECFP" refers to enhanced cyan fluorescent protein.

As used herein, the term "NFP" refers to novel fluorescent protein, and the term "CNFP" refers to cyan novel fluorescent protein.
10 Specifically, "CNFP" refers to amFP486.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis,
15 Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell
20 Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about
25 the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not

limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5'

direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when

the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct where
5 portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding
10 sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter
15 code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine; X: any residue). NH₂ refers to the free amino group present at the amino
20 terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-59 is used.

The present invention is directed to an isolated DNA
25 selected from the group consisting of: (a) isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the

degeneracy of the genetic code, and which encodes a fluorescent protein. Preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 61, and the fluorescent protein has the amino acid sequence shown in SEQ ID No. 56. More preferably, the DNA is amFP486, Mut15 or Mut32, or humanized version.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising the DNA and regulatory elements necessary for expression of the DNA in the cell. Specifically, the DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56. Preferably, the vector is constructed by amplifying the DNA and then inserting the amplified DNA into EGFP-N1 backbone, or by fusing different mouse ODC degradation domains such as d1, d2 and d376 to the C-terminal of the DNA and then inserting the fusion DNA into EGFP-N1 backbone.

In still another embodiment of the present invention, there is provided a host cell transfected with the vector of the present invention, which expresses a fluorescent protein of the present invention. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells and insect cells. A representative example of mammalian cell is HEK 293 cell and an example of bacterial cell is an *E. coli* cell.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of:

- (a) an isolated DNA which encodes a fluorescent protein, wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence;
- (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein;
- and (c) an isolated DNA differing from the isolated DNAs of

(a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the organism is from Sub-order Endomyaria. Even more preferably, the
5 organism is from Family Actiniidae, Genus Anemonia. Most preferably, the organism is *Anemonia majano*.

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) an isolated protein encoded by a DNA which encodes
10 a fluorescent protein wherein the DNA is from an organism from Class Anthozoa and wherein the organism does not exhibit bioluminescence; (b) an isolated protein encoded by a DNA which hybridizes to isolated DNA of (a); and (c) an isolated protein encoded by a DNA differing
15 from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code. Preferably, the isolated and purified fluorescent protein is amFP486.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by
20 means of hybridization, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16 and is used as a primer in polymerase chain reaction. Alternatively, it can be used as
25 a probe for hybridization screening of the cloned genomic or cDNA library.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 15 **Biological Material**

Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

TABLE 1Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp. "green"	Western Pacific	green spots on oral disk
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthesized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2Oligos Used in cDNA Synthesis and RACE

- 5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)
- T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGAGTCGACCG(T)₁₃
(SEQ ID No. 17)
- 10 TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)
- T7-TS:
15 5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)
- T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)
- 20 TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

25

EXAMPLE 3**Oligo Design**

To isolate fragments of novel fluorescent protein cDNAs,
5 PCR using degenerate primers was performed. Degenerate primers
were designed to match the sequence of the mRNAs in regions that
were predicted to be the most invariant in the family of fluorescent
proteins. Four such stretches were chosen (Table 3) and variants of
degenerate primers were designed. All such primers were directed to
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2
shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5) GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4Isolation of 3'-cDNA Fragments of nEPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 µl of 20-fold
 10 dilution of the amplified cDNA sample was added into the reaction
 mixture containing 1X Advantage KlenTaq Polymerase Mix with
 provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel
5 fluorescent protein cDNAs, two nested 5'-directed primers were
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were
then amplified using two consecutive PCRs. In the next PCR reaction,
the novel approach of "step-out PCR" was used to suppress background
amplification. The step-out reaction mixture contained 1x Advantage
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer
(CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer
(see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of
T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the
amplified cDNA sample in a total volume of 20 μ l. The cycling profile
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was diluted 50-fold in water and one μ l of this dilution
was added to the second (nested) PCR. The reaction contained 1X
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),
20 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M
of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was then cloned into pAtlas vector (CLONTECH) according
25 to the manufacturer's protocol.

TABLE 5Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCTT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of NFPs in *E.coli*

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 10 6). Primers with SEQ ID Nos. 36 and 37 were the primers used to prepare the am486 DNA. Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading 15 frames of the vector-encoded 6xHis-tag and NFP. The PCR was performed as follows: 1 μ l of the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.2 μ M of upstream primer and 0.2 μ M of downstream 20 primer, in a final total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction 25 endonucleases corresponding to the primers' sequence according to standard protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium

(supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 µg/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and
5 incubation was allowed to proceed at 37°C for another 16 hours. The cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nEPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' - <u>acatggatccgctctttcaaaca</u> agtttatc (SEQ ID No. 36) BamHI	5' -tagtactcgagcttattcgta tttcagtgaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5' - <u>acatggatccaacattttttga</u> gaaacg (SEQ ID No. 38) BamHI S: 5' - <u>acatggatccaaagctctaacc</u> accatg (SEQ ID No. 39) BamHI	5' -tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5' - <u>acatggatccgctcagtc</u> aaag cacggt (SEQ ID No. 41) BamHI	5' -tagtactcgagggttggaactaca tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5' - <u>acatggatccaggtcttccaagaat</u> gttatc (SEQ ID No. 43) BamHI	5' -tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5' - <u>acatggatccagttggtccaagagtgtg</u> (SEQ ID No. 45) BamHI	5' -tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5' - <u>acatggatccgcttccttttaagaagact</u> (SEQ ID No. 47) BamHI	5' -tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5' - <u>acatggatccagttgtccaagaatgtgat</u> (SEQ ID No. 49) BamHI	5' -tagtactcgaggccattacg ctaate (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5' - <u>acatggatccagtgcaactaaagaagaatg</u> (SEQ ID No. 51)	5' -tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7Novel Fluorescent Proteins and cDNAs Encoding the Proteins

One of the full-length cDNAs encoding novel fluorescent proteins is described herein (amFP486). The nucleic acid sequence and deduced amino acid sequence are SEQ ID Nos. 55 and 56, respectively. The spectral properties of amFP486 are listed in Table 7, and the emission and excitation spectrum for amFP486 is shown in Figure 2.

TABLE 7Spectral Properties of the Isolated amFP486

Species:	Anemonia majano	Max. Extinction Coefficient:	40,000
nFP Name:	amFP486	Quantum Yield	0.24
Absorbance Max. (nm):	458	Relative Brightness:*	0.43
Emission Max. (nm):	486		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 8Construction of amFP486 Mutants

Two mutants of amFP486 were generated, Mut15 and Mut32. Mut15 has the nucleic acid sequence shown in SEQ ID No. 57. Compared with wildtype amFP486, Mut15 has the following point

mutations: A to G at position 101 (numbered from beginning of ATG); T to C at position 129; AAA to TTG at positions 202-204; C to T at position 240. Table 8 lists the spectral properties of Mut15 and Mut32.

5

TABLE 8Spectral Properties of the Isolated Mut15 and Mut32

	Species:	Anemonia majano	Max. Extinction Coefficient:	53,400
10	nFP Name:	Mut15	Quantum Yield	0.32
	Absorbance Max. (nm):	460	Relative Brightness:*	0.78
15	Emission Max. (nm):	485		
	Species:	Anemonia majano	Max. Extinction Coefficient:	36,000
	nFP Name:	Mut32	Quantum Yield	0.42
20	Absorbance Max. (nm):	466	Relative Brightness:*	0.69
	Emission Max. (nm):	488		

25 *relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 930 Construction and Functional Analysis of Vectors

Mut32 DNA was amplified via PCR and reconstructed to EGFP-N1 backbone with BamHI and NotI restriction enzyme sites. This

vector has the same multiple cloning sites as EGFP-N1. The nucleic acid sequence of the vector (pCNFPMut32-N1) is shown in SEQ ID No. 58.

Functional test of the generated vectors was performed by transient transfection in 293 cells. After 24-hour expression, brighter
5 fluorescent intensity and less photobleaching of pCNFPMut32-N1 were observed by microscopy when compared with pECFP-N1 side by side (Figures 3A and 3B).

Mut32 has fast folding and bright fluorescent intensity, which makes it useful for number of applications. Some fusion
10 proteins were tested, such as PKC-gamma-CNFP. PKC was observed to translocate from cytosol to the plasma membrane when cells were treated with PMA (phorbol 12-myristate 13-acetate). Figure 4 shows control and PMA-treated cells.

15

EXAMPLE 10

Generation of Destabilized amFP486 Vectors as Transcription Reporters

Since amFP486 is very stable, it is necessary to generate
20 destabilized versions of amFP486 in order to observe the rapid turnover of the protein. By using the same technology for destabilized EGFP, three destabilized amFP486 vectors were constructed by fusing different mouse ODC degradation domains such as d1, d2 and d376 to the C-terminal of wild type amFP486. The sequences for vectors pCNFP-
25 MODCd1 and pCNFP-MODCd2 are shown in SEQ ID No. 59 and SEQ ID No. 60, respectively. The vectors were constructed in EGFP-N1 backbone.

Vectors of pCRE-d1CNFP and pNF- κ B-d1CNFP were constructed by placing d1CNFP downstream of cAMP response element

(CRE) or NF- κ B response element, respectively. Expression of d1CNFP is up-regulated upon activation of these response elements.

5

EXAMPLE 11

Functional Analysis of Destabilized amFP486

Functional test of the destabilized amFP486 was performed by transient transfection in 293 cells. After 24-hour expression, the
10 fluorescent intensity was decreased gradually from d2, d1 and d376 because of the fusion with different mouse ODC degradation domains. After 4-hour treatment with protein synthesis inhibitor cycloheximide, d2 fluorescent intensity did not change very much; however, d1 fluorescent intensity decreased further 50% of its original intensity
15 (Figures 5A and 5B). The half-life of d1 is around 4 hours.

MODCd1 is a valuable tool for application as a transcription reporter. However, compared with EGFP-d1 (1-hour half-life), pCNFP-MODCd1 half-life (4 hours) is still long, so further mutagenesis for MODC degradation domain is still needed for shorter half-life version.

20 Functional test of vectors pCRE-d1CNFP and pNF- κ B-d1CNFP was performed by transient transfection in HEK 293 cells. 16 hours post transfection, 10 μ m forskolin was added to induce CRE and 100 ng/ml TNF-alpha was added to induce NF- κ B for 6 hours. Expression of d1CNFP was analysed using FACS Calibur. Up to 7 fold increase of
25 fluorescence in forskolin induced CRE activation and 4 fold increase of fluorescence in TNF-alpha induced NF-KB activation was observed (data not shown).

EXAMPLE 12

Construction and Functional Test for Humanized Mut32 (phCNEP-N1)

5 Since mammalian expression is a very popular tool, human favored codon version is needed for better expression in mammalian cells. To generate humanized Mut32, the Mut 32 sequence was first changed to human favored codon and 23 oligos (12F and 11R) were designed. Next, four rounds of PCR amplification were performed, each
10 round for 20 cycles. PCR cycle was designed as follows: 94°C for 1 min; 94°C for 1 min; 40°C for 1 min; and 72°C for 1 min. The four rounds were: for 1st round, mixing 2 µl each of every 4 oligos (60 bp), 5 µl buffer, 1 µl pfu, 1 µl dNTP to make total volume of 50 µl. After 20 cycles of PCR, 5 sets of 150 bp and 1 set of 4 last oligos of 90 bp
15 products were obtained. For 2nd round, mixing new crude PCR products 10 µl each, 5 µl buffer, 1 µl pfu, 1 µl dNTP to make total volume of 50 µl. After 20 cycles of PCR, 2 sets of 270 bp and 1 set of 210 bp PCR products were obtained. For 3rd round, mixing new crude PCR products. After 20 cycles of PCR, 1 set of 510 bp and 1 set of 450 bp
20 products were obtained. For 4th round, mixing new crude products. After 20 cycles of PCR, final PCR product (690 bp) was obtained. Further PCR amplification was performed using 1F and 11R primers.

 As a result, humanized Mut32 was generated, having the sequence shown in SEQ ID No. 61. This humanized Mut32 was
25 constituted into EGFP-N1 backbone.

EXAMPLE 13

Expression of Wildtype and Mutant amFP486 in Mammalian Cells

5 The original plasmid amFP486 DNAs (wildtype, Mut15 and Mut32 in pQE30) were used to construct N1 version of amFP486 wildtype, Mut15 and Mut32 as described in Example 9. The DNAs were inserted into *E.coli* DH5 α . HEK 293 cells were transferred with each of the three N1 constructs using Calcium Phosphate method (Clontech
10 product #K2051-1).

 The fluorescent intensity of the transfected cells was analyzed on FACS using FL1 (510/30) detecting channel. Five samples were analyzed in parallel for each construct. The histograms of all the analysis are shown in Figures 6-8.

15 The M1 gate is set as shown on the histograms. The mean value of FL1 fluorescent intensity of the M1 population of each sample is summarized in Table 9. It shows that the average of the mean value of each construct (Wildtype, Mut15, and Mut32) has no significant difference.

20

TABLE 9FL1 Fluorescent Intensity of M1 Population

Sample #	Wildtype (Figure 6A-6E)	Mut15 (Figure 7A-7E)	Mut32 (Figure 8A-8E)
1	82.84	106.95	84.51
2	77.52	108.73	91.41
3	111.85	97.08	91.30
4	113.06	90.16	98.16
5	104.95	86.34	111.44
Mean	98.04	97.85	95.36

5

EXAMPLE 14Generation and Expression of Fusion Protein Mut15-mdm2

10 The Mut15-mdm2 fusion was generated by the following steps: first, mdm2 DNA was obtained by amplifying human Marathon cDNA library (Burke's Lymphoma) using primers ATGTGCAATACCAACATGTCTGTACC (SEQ ID No. 62) and CTAGGGGA AATAAGTTAGCAC (SEQ ID No. 63); secondly, the purified PCR product

15 was then amplified with primers GGAATTCCAGCCATGGTGTG CAATACCAACATGTCTGTACC (SEQ ID No. 64) and TCCCCCGGGGGGAA ATAAGTTAGCAC (SEQ ID No. 65) in order to add Kozac sequence and restriction sites; thirdly, the purified PCR product from step 2 was

20 digested with EcoR I and Sma I and inserted into EcoR I and SmaI of NFP1Mut15-N1 vector (this vector was generated using BamH I and Not I sites of the pEGFP-N1 backbone).

The generated Mut15-mdm2 fusion was then expressed in HEK293 cells. Figure 9 shows the results.

5

EXAMPLE 15

Comparison of the Protein Fluorescent Intensity

PQE30 amFP486 wildtype, Mut15 and Mut32 were transformed into DH5 α . The bacteria grew in the presence of 1 mM IPTG overnight to induce the protein expression. Cells were lysed in 100 mM Tris, pH8.0 by sonication. Cell lysate was collected after centrifuge at 3000 rpm for 15 minutes at room temperature. The proteins were purified with TALON Metal Affinity Resin. Briefly, after the protein was absorbed on the resin, the beads were washed in stepwise with first wash, then first elution (50 mM imidazole) and second elution (200 mM imidazole) in 100 mM Tris-HCl, pH 8.0. The protein is found mostly in the second step elution. It was found that Mut32 has the highest bacterial expression level, while Mut15 has the lowest.

Samples of each elution fraction were run on SDS-PAGE to check the purity of the proteins. Both wildtype amFP486 and Mut32 show a single band, while Mut15 has two more minor bands with higher molecular weight (data not shown).

The protein concentration (fractionII-2) was checked and measured by Bradford assay (Bio-Rad standard assay) using BSA as a standard. The spectra are shown in Figures 10-13. The fluorescence intensity (fraction II-2) was determined with LS50B Luminescence Spectrometer LS50B. EX = 458 nm, EM = 492 nm, both slits = 2.5 nm. Table 10 shows the protein concentration, relative fluorescent (FL)

intensity and intensity/ μg protein in 700 μl volume. It shows that Mut32 is as bright as wildtype, while Mut15 is worse than the wildtype.

5

TABLE 10

	Protein Concentration	Relative FL Intensity	Intensity/ μg Protein in 700 μl Volume
Wildtype II-2	1.26 $\mu\text{g}/5 \mu\text{l}$	37.805/5 μl	30.00
Mut15II-2	0.64 $\mu\text{g}/5 \mu\text{l}$	10.152/5 μl	15.86
Mut32II-2	6.17 $\mu\text{g}/5 \mu\text{l}$	186.474/5 μl	30.22

Any patents or publications mentioned in this specification
10 are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present
15 invention is adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects and ends inherent therein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein, are presently representative of preferred embodiments, are exemplary, and
20 are not intended as limitations on the scope of the invention. Changes to the methods and compounds, and other uses, will occur to those skilled in the art and are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

5 (a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from a Class Anthozoa and wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

10 (c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

2. The DNA sequence of claim 1, wherein said organism
15 is from Sub-class Zoantharia.

3. The DNA sequence of claim 2, wherein said organism is from Order Actiniaria.

20 4. The DNA sequence of claim 3, wherein said organism is from Sub-order Endomyaria.

5. The DNA sequence of claim 4, wherein said organism is from Family Actiniidae.

25 6. The DNA sequence of claim 5, wherein said organism is from Genus Anemonia.

7. The DNA sequence of claim 6, wherein said organism is *Anemonia majano*.

8. A DNA sequence encoding a fluorescent protein
5 selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein having a nucleotide sequence selected from the group consisting of SEQ ID Nos. 55, 57, and 61;

(b) an isolated DNA which hybridizes to isolated DNA of
10 (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code, and which encodes a fluorescent protein.

15 9. The DNA sequence of claim 8, wherein said DNA encodes a fluorescent protein having an amino acid sequence shown in SEQ ID No. 56.

10. The DNA sequence of claim 8, wherein said DNA is
20 selected from the group consisting of amFP486, Mut15 and Mut32.

11. The DNA sequence of claim 8, wherein said DNA is humanized DNA.

25 12. A vector capable of expressing the DNA sequence of claim 1 in a recombinant cell, wherein said vector comprising said DNA and regulatory elements necessary for expression of the DNA in the cell.

13. The vector of claim 12, wherein said DNA encodes a fluorescent protein having the amino acid sequence shown in SEQ ID No. 56.

5 14. The vector of claim 12, wherein said vector is constructed by amplifying said DNA and then inserting the amplified DNA into EGFP-N1 backbone.

15 15. The vector of claim 14, wherein said DNA is selected
10 from the group consisting of amFP486, Mut15 and Mut32.

16. The vector of claim 14, wherein said DNA is humanized DNA.

15 17. The vector of claim 12, wherein said vector is constructed by fusing different mouse ODC degradation domains to the C-terminal of said DNA and then inserting the fusion DNA into EGFP-N1 backbone.

20 18. The vector of claim 17, wherein said mouse ODC degradation domains are selected from the group consisting of d1, d2 and d376.

19. The vector of claim 17, wherein said DNA is selected
25 from the group consisting of non-humanized and humanized DNA.

20. A host cell transfected with the vector of claim 12, wherein said cell is capable of expressing a fluorescent protein.

21. The host cell of claim 20, wherein said cell is selected from the group consisting of bacterial cells, mammalian cells, plant cell, yeast and insect cells.

5 22. The host cell of claim 21, wherein said mammalian cell is HEK 293 cell.

23. The host cell of claim 21, wherein said bacterial cell is an *E. coli* cell.

10

24. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

15 (a) an isolated DNA which encodes a fluorescent protein from an organism from Class Anthozoa, wherein said organism does not exhibit bioluminescence;

 (b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

 (c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic
20 code and which encodes a fluorescent protein.

25 25. The isolated and purified fluorescent protein of claim 24, wherein said organism is from Sub-class Zoantharia.

26. The isolated and purified fluorescent protein of claim 25, wherein said organism is from Order Actiniaria.

27. The isolated and purified fluorescent protein of claim 26, wherein said organism is from Sub-order Endomyaria.

28. The isolated and purified fluorescent protein of claim 27, wherein said organism is from Family Actiniidae.

5 29. The isolated and purified fluorescent protein of claim 28, wherein said organism is from Genus *Anemonia*.

30. The isolated and purified fluorescent protein of claim 29, wherein said organism is *Anemonia majano*.

10

31. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) isolated DNA which encodes a fluorescent protein having an amino acid sequence shown in SEQ ID No. 56;

15 (b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) isolated DNA differing from said isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

20

32. The isolated and purified fluorescent protein of claim 31, wherein said protein is amFP486.

33. An amino acid sequence which can be used as a basis
25 for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridization, wherein said sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14.

34. The amino acid sequence of claim 26, wherein said oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.

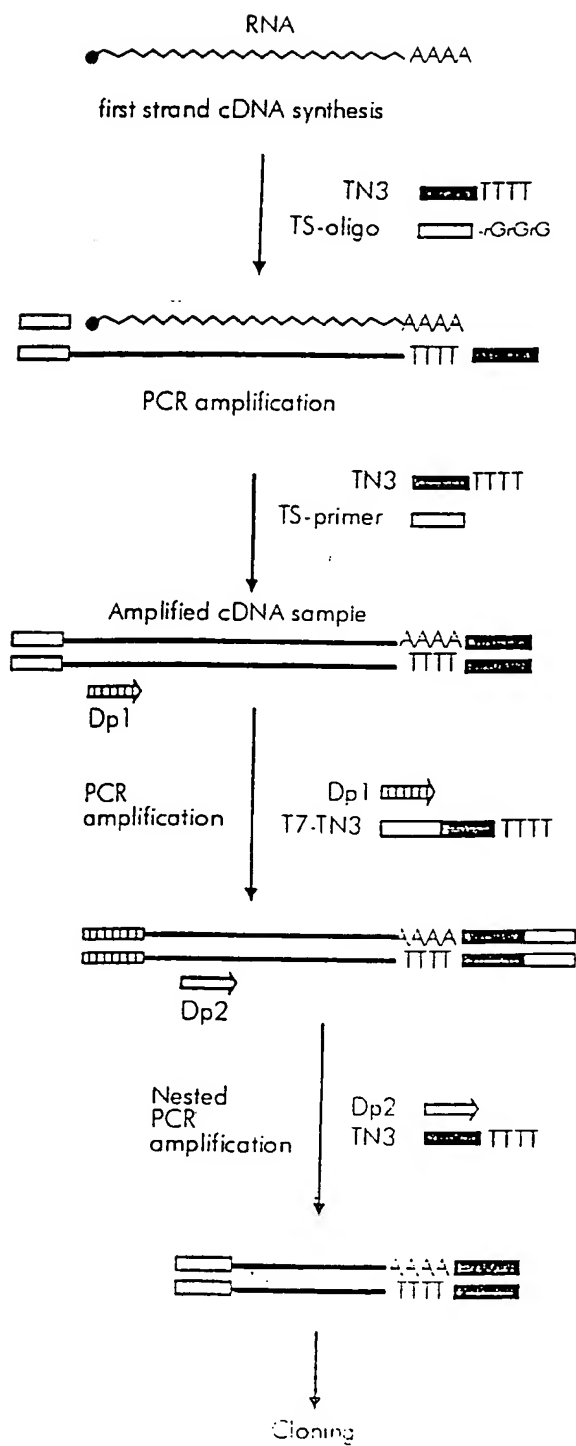


Figure 1

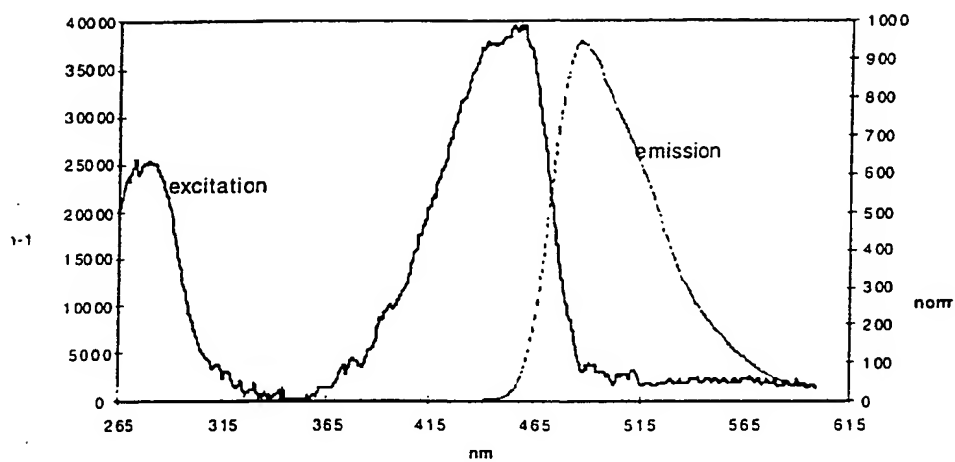


Figure 2

B. pECFP-N1



A. pCNEP-N1



Figure 3 :

Traci Yerby, 12/9/99 12:.. PM -0800, NFP1 Data

Date: Thu, 09 Dec 1999 12:17:31 -0800

From: "Traci Yerby" <TRYERBY@CLONTECH.COM>

To: <baadler@flash.net>

Subject: NFP1 Data

Mime-Version: 1.0

Hi Ben,

This should be the last of it.

Data for PMA treated cells (FIG 8)

Traci

Content-Type: application/octet-stream; name="PKCr-NCFP.psd"

Content-Disposition: attachment; filename="PKCr-NCFP.psd"

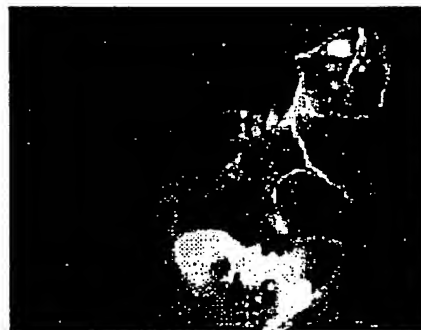
PKC γ -NCFP translocation

Fig. 4A



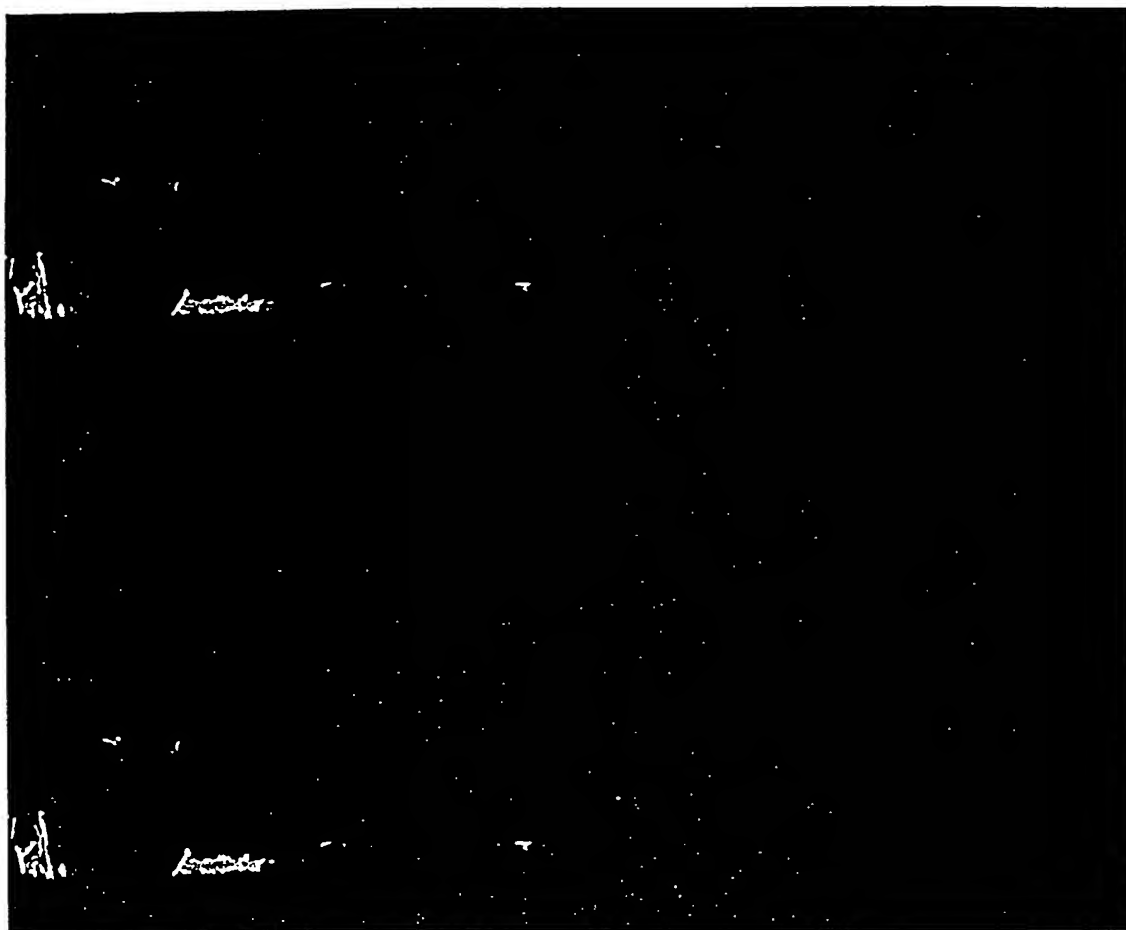
control

Fig. 4B



PMA treated

Figure 5A

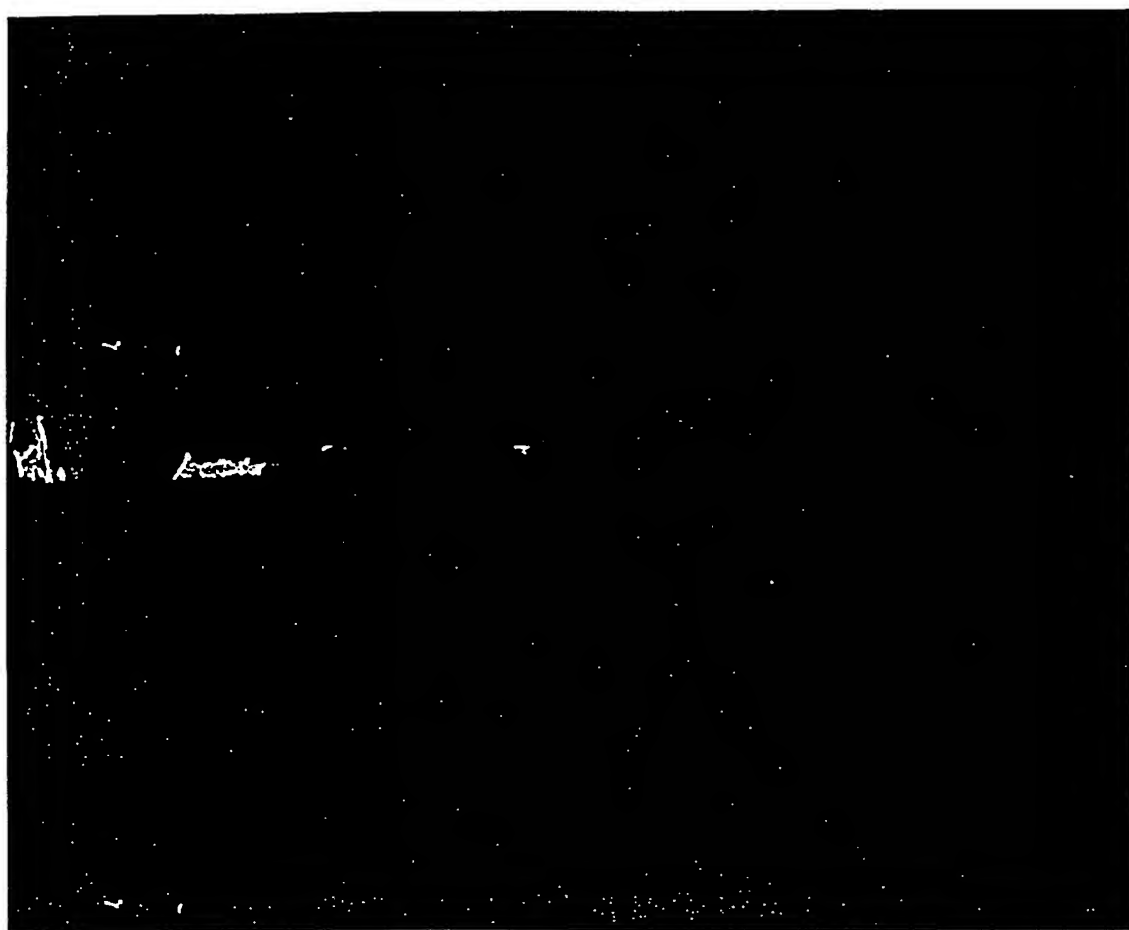


pcNFP-MODC1 control

picture 2

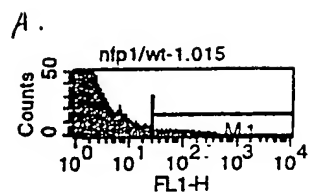
provision sample, should be seen

Figure 5B



PCNFP-mucic(1) 4h + cycloheximide

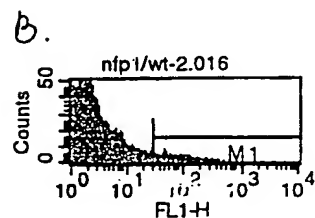
picture 2
pseudocolor plot



File: nfp1/wt-1.015

Acquisition Date: 12-Aug-99

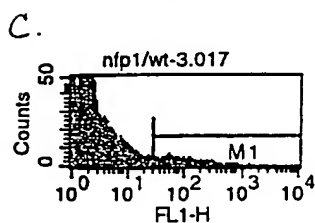
Marker	% Total	Mean	Geo Mean
All	100.00	5.19	2.13
M1	3.07	82.84	66.13



File: nfp1/wt-2.016

Acquisition Date: 12-Aug-99

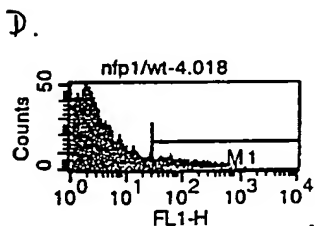
Marker	% Total	Mean	Geo Mean
All	100.00	5.49	2.17
M1	3.73	77.52	62.95



File: nfp1/wt-3.017

Acquisition Date: 12-Aug-99

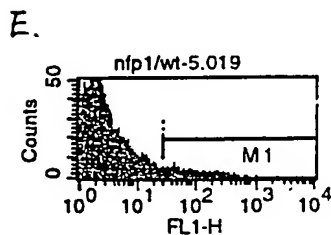
Marker	% Total	Mean	Geo Mean
All	100.00	7.75	2.31
M1	4.57	111.85	80.87



File: nfp1/wt-4.018

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	8.56	2.43
M1	5.16	113.06	82.26



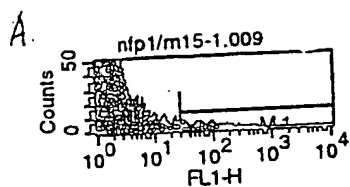
File: nfp1/wt-5.019

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.41	2.22
M1	3.61	104.95	77.54

Figure 6

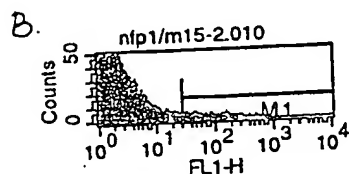
8/14



File: nfp1/m15-1.009

Acquisition Date: 12-Aug-99

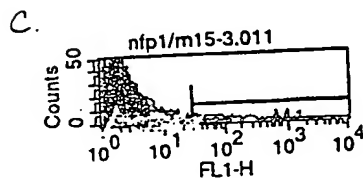
Marker	% Total	Mean	Geo Mean
All	100.00	5.74	2.17
M1	2.94	106.95	80.29



File: nfp1/m15-2.010

Acquisition Date: 12-Aug-99

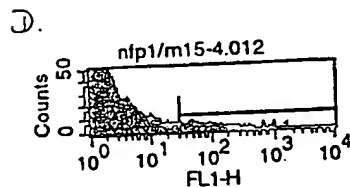
Marker	% Total	Mean	Geo Mean
All	100.00	6.57	2.24
M1	3.66	108.73	78.64



File: nfp1/m15-3.011

Acquisition Date: 12-Aug-99

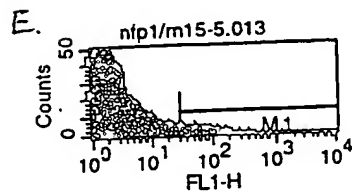
Marker	% Total	Mean	Geo Mean
All	100.00	6.26	2.24
M1	3.70	97.08	73.22



File: nfp1/m15-4.012

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.83	2.28
M1	4.65	90.16	69.85

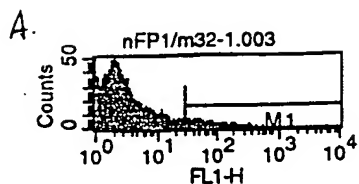


File: nfp1/m15-5.013

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	6.32	2.31
M1	4.16	86.34	67.43

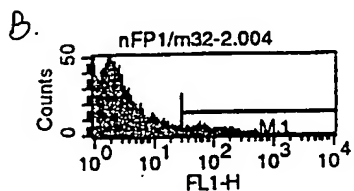
Figure 7



File: nFP1/m32-1.003

Acquisition Date: 12-Aug-99

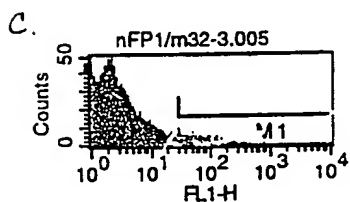
Marker	% Total	Mean	Geo Mean
All	100.00	5.55	2.08
M1	3.51	84.51	67.46



File: nFP1/m32-2.004

Acquisition Date: 12-Aug-99

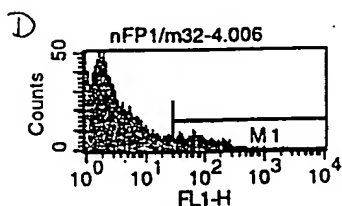
Marker	% Total	Mean	Geo Mean
All	100.00	6.30	2.13
M1	4.09	91.41	69.57



File: nFP1/m32-3.005

Acquisition Date: 12-Aug-99

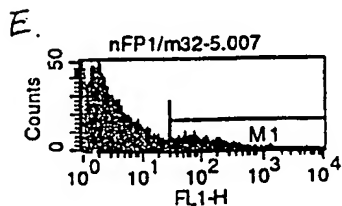
Marker	% Total	Mean	Geo Mean
All	100.00	6.44	2.20
M1	4.16	91.30	70.91



File: nFP1/m32-4.006

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	7.64	2.21
M1	5.15	98.16	71.62

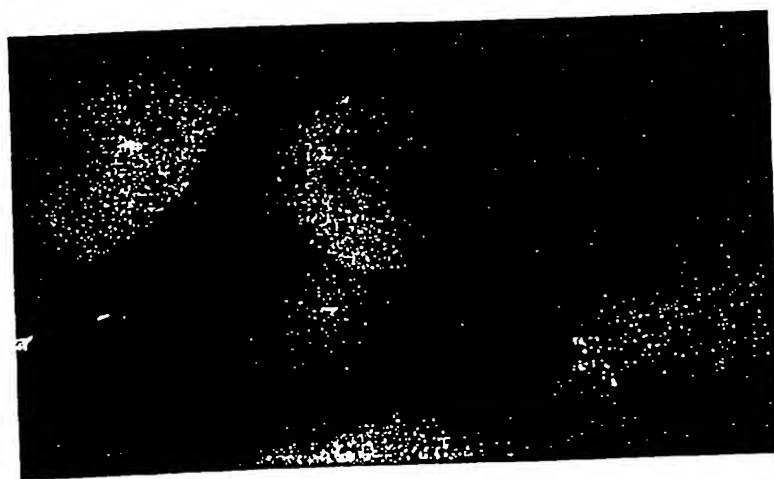


File: nFP1/m32-5.007

Acquisition Date: 12-Aug-99

Marker	% Total	Mean	Geo Mean
All	100.00	10.07	2.41
M1	6.74	111.44	78.24

Figure 8



Fusion Mdm2-nFP1mut15

Figure 9

Date: 9/8/99

Time: 12:01:36 PM

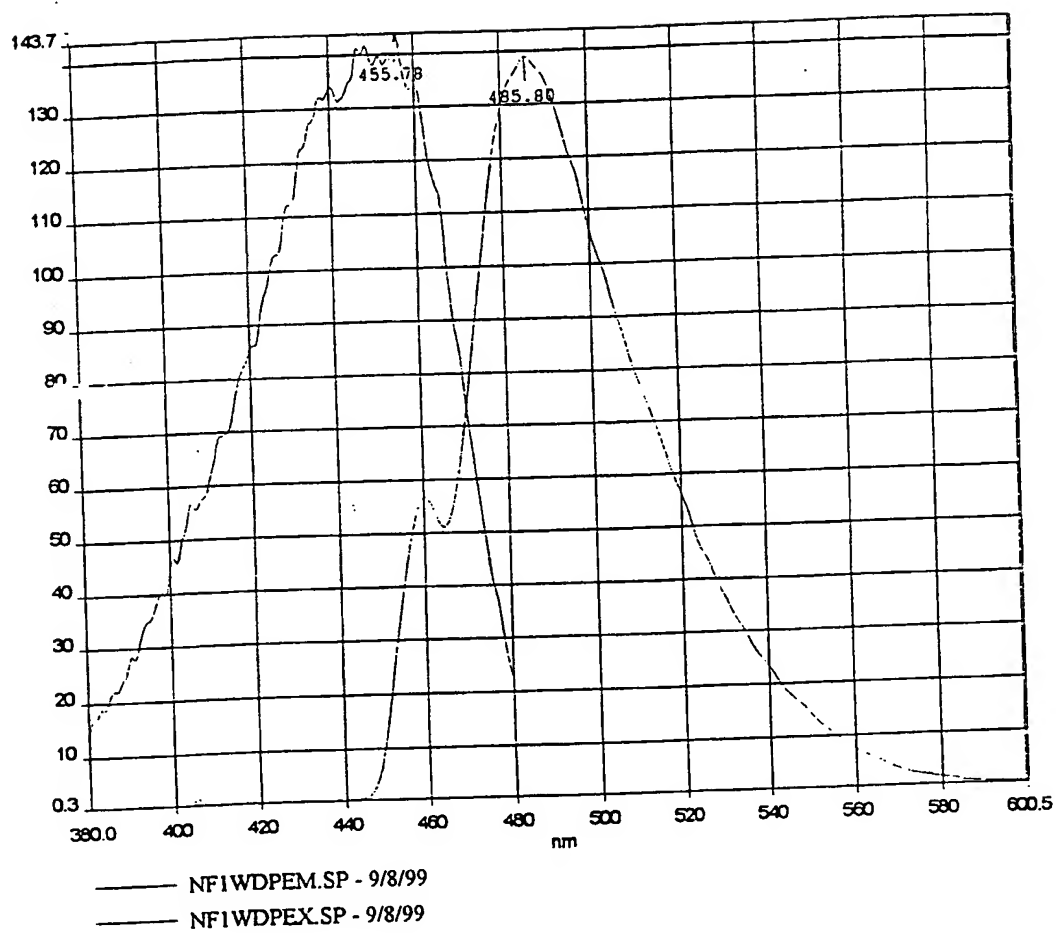


Figure 1D

Date: 9/8/99

Time: 12:05:19 PM

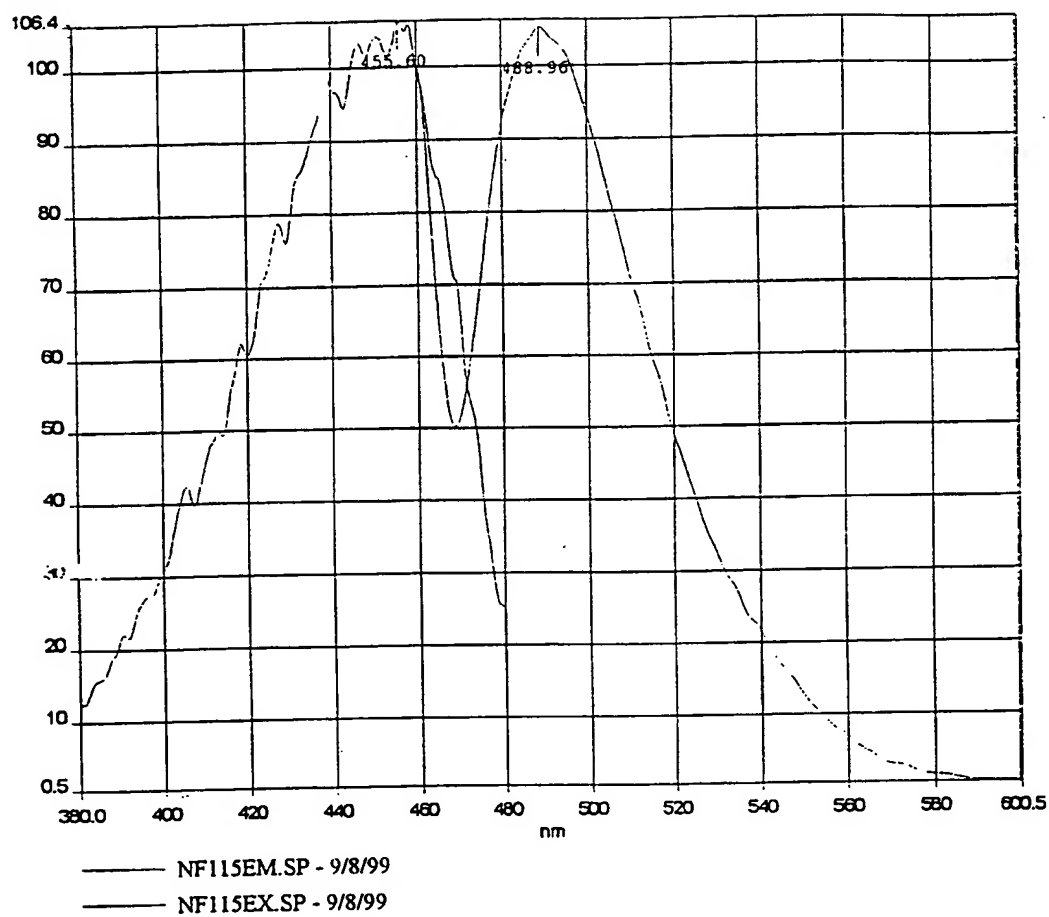


Figure 11

Date: 9/8/99

Time: 12:09:58 PM

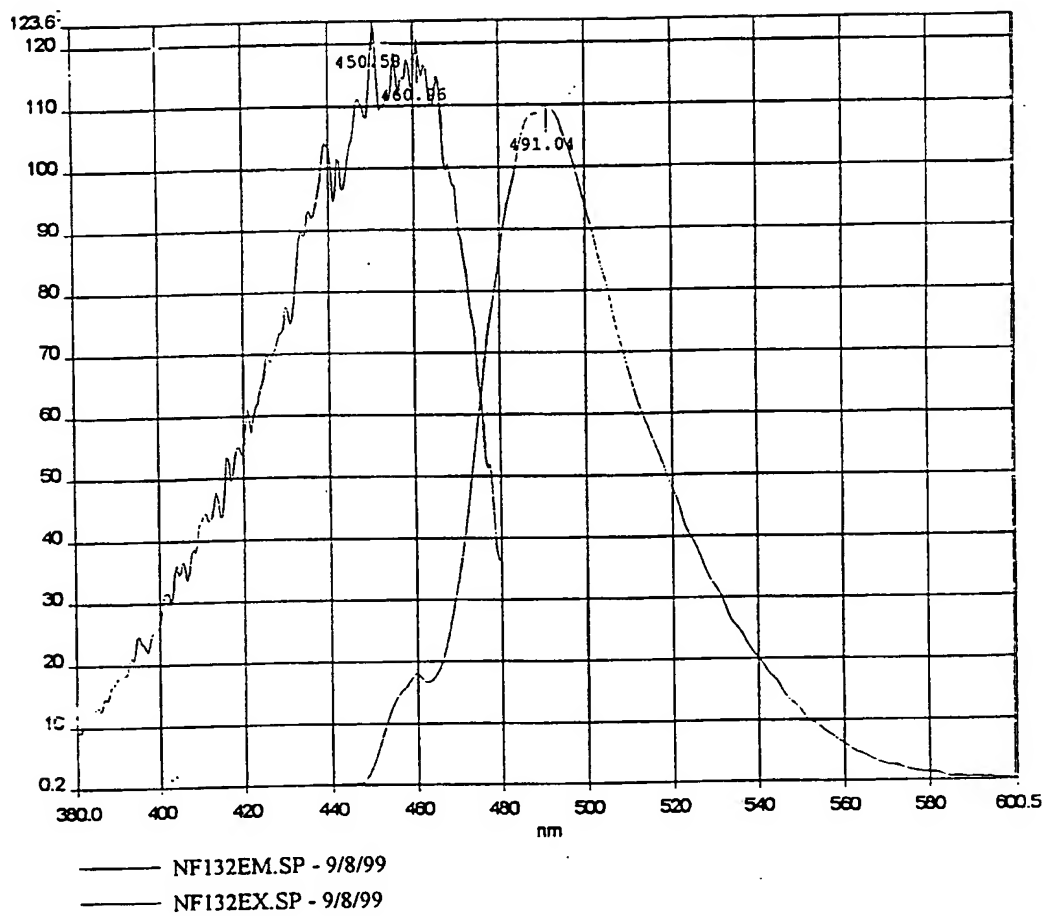


Figure 12

14/14

Date: 9/8/99

Time: 11:44:24 AM

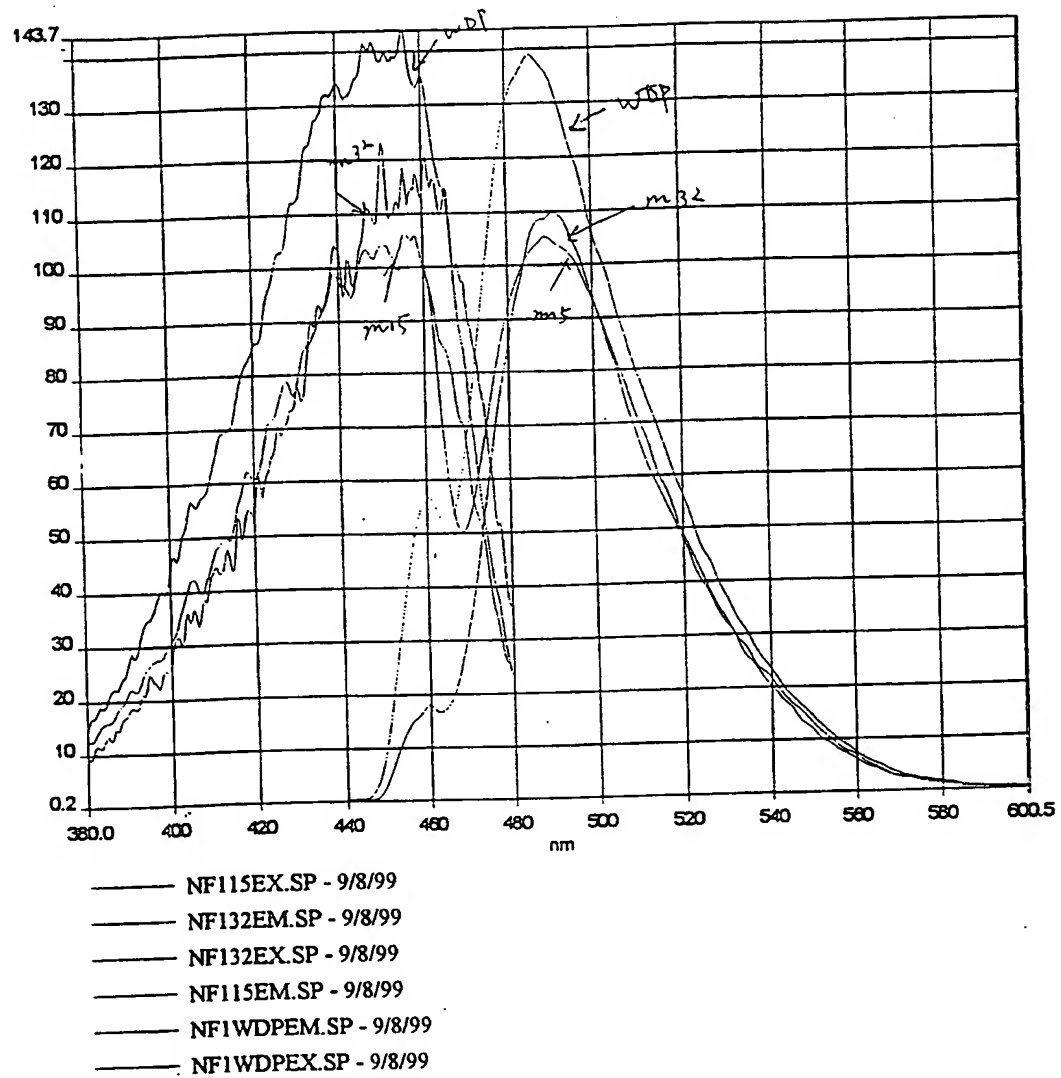


Figure 13

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
 Labas, Yulii A.
 Matz, Mikhail V.
 5 Fradkov, Arcady F.
 Chen, Ying
 Hu, Lanrong
 Ding, Li
 Fang, Yu
 10 <120> Fluorescent proteins from non-bioluminescent
 species of Class Anthozoa, genes encoding such
 proteins and uses thereof
 <130> D6196D1/PCT
 <141> 1999-12-09
 15 <150> 09/210,330
 <151> 1998-12-11
 <160> 65
 <210> 1
 20 <211> 25
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 25 <223> primer TN3 used in cDNA synthesis and RACE
 <400> 1
 cgagctcgac cgtttttttt ttttt 25
 <210> 2
 30 <211> 23
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 35 <223> primer TS used in cDNA synthesis and RACE
 <400> 2
 aagcagtggg atcaacgcag agt 23

5 <210> 3
 <211> 6
 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <222> 21
 <223> amino acid sequence of a key stretch on which
 10 represents primer NGH is based; Xaa at position 21
 unknown
 <400> 3
 Gly Xaa Val Asn Gly His
 5
 15 <210> 4
 <211> 20
 <212> DNA
 <213> artificial sequence
 20 <220>
 <221> primer_bind
 <222> 12
 <223> primer NGH used for isolation of fluorescent
 protein; n at position 12 represents any of the
 25 four bases
 <400> 4
 gayggctgcg tnaayggdca 20

 30 <210> 5
 <211> 5
 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <222> 31...35
 35 <223> amino acid sequence of a key stretch on which
 primers GEGa and GEGb are based
 <400> 5

Gly Glu Gly Glu Gly
5

5 <210> 6
 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 10 <223> primer GEGa used for isolation of fluorescent
 protein
 <400> 6

gttacaggtg arggmgargg 20

15 <210> 7
 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 20 <221> primer_bind
 <223> primer GEGb used for isolation of fluorescent
 protein
 <400> 7

gttacaggtg arggkgargg 20

25 <210> 8
 <211> 5
 <212> PRT
 <213> *Aequorea victoria*
 30 <220>
 <222> 31...35
 <223> amino acid sequence of a key stretch on which
 primers GNGa and GNGb are based
 <400> 8

35 Gly Glu Gly Asn Gly
5

<210> 9
 <211> 20
 <212> DNA
 <213> artificial sequence
 5 <220>
 <221> primer_bind
 <223> primer GNGa used for isolation of fluorescent
 protein
 <400> 9

10 gttacaggtg arggmaaygg 20

<210> 10
 <211> 20
 <212> DNA
 15 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer GNGb used for isolation of fluorescent
 protein
 20 <400> 10

gttacaggtg arggkaaygg 20

<210> 11
 <211> 5
 25 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <222> 127...131
 <223> amino acid sequence of a key stretch on which
 30 primer NFP is based
 <400> 11

Gly Met Asn Phe Pro
5

35 <210> 12
 <211> 5
 <212> PRT

<213> *Aequorea victoria*
<220>
<222> 127...131
<223> amino acid sequence of a key stretch on which
5 primer NFP is based
<400> 12

Gly Val Asn Phe Pro

5

10 <210> 13
<211> 20
<212> DNA
<213> artificial sequence
<220>
15 <221> primer_bind
<223> primer NFP used for isolation of fluorescent
protein
<400> 13

ttccayggtr tgaayttycc

20

20

<210> 14
<211> 4
<212> PRT
<213> *Aequorea victoria*
25 <220>
<222> 134...137
<223> amino acid sequence of a key stretch on which
primers PVMa and PVMb are based
<400> 14

30 Gly Pro Val Met

<210> 15
<211> 21
35 <212> DNA
<213> artificial sequence

<220>
<221> primer_bind
<222> 15
<223> primer PVMa used for isolation of fluorescent
5 protein; n at position 15 represents any of the
four bases
<400> 15
cctgccrayg gtcngtmat g 21

10 <210> 16
<211> 21
<212> DNA
<213> artificial sequence
<220>
15 <221> primer_bind
<222> 15
<223> primer PVMb used for isolation of fluorescent
protein; n at position 15 represents any of the
four bases
20 <400> 16
cctgccrayg gtcngtkat g 21

<210> 17
<211> 47
25 <212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> primer T7-TN3 used in cDNA synthesis and RACE
30 <400> 17
gtaatacgac tcactatagg gccgcagtcg accgtttttt tttttt
47

<210> 18
35 <211> 45
<212> DNA
<213> artificial sequence

<220>
 <221> primer_bind
 <223> primer T7-TS used in cDNA synthesis and RACE
 <400> 18

5 gtaatacgac tcactatagg gcaagcagtg gatatcaacgc agagt
 45

<210> 19
 <211> 22
 10 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer T7 used in cDNA synthesis and RACE
 15 <400> 19

gtaatacgac tcactatagg gc 22

<210> 20
 <211> 21
 20 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
 25 *Anemonia majano*
 <400> 20

gaaatagtca ggcataactgg t 21

<210> 21
 30 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 35 <223> gene-specific primer used for 5'-RACE for
Anemonia majano

<400> 21
 gtcaggcata ctggtaggat 20

 <210> 22
 5 <211> 21
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 10 <223> gene-specific primer used for 5'-RACE for
Clavularia sp.
 <400> 22
 cttgaaatag tctgctatat c 21

 15 <210> 23
 <211> 19
 <212> DNA
 <213> artificial sequence
 <220>
 20 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Clavularia sp.
 <400> 23
 tctgctatat cgtctgggt 19

 25 <210> 24
 <211> 23
 <212> DNA
 <213> artificial sequence
 <220>
 30 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Zoanthus sp.
 <400> 24
 35 gttcttgaaa tagtctacta tgt 23

	<210>	25
	<211>	20
	<212>	DNA
5	<213>	artificial sequence
	<220>	
	<221>	primer_bind
	<223>	gene-specific primer used for 5'-RACE for <i>Zoanthus sp.</i>
10	<400>	25

gtctactatg tcttgaggat 20

	<210>	26
	<211>	19
15	<212>	DNA
	<213>	artificial sequence
	<220>	
	<221>	primer_bind
	<223>	gene-specific primer used for 5'-RACE for
20		<i>Discosoma</i> sp. "red"
	<400>	26

caagcaaattg gcaaaggctc 19

	<210>	27
25	<211>	19
	<212>	DNA
	<213>	artificial sequence
	<220>	
	<221>	primer_bind
30	<223>	gene-specific primer used for 5'-RACE for <i>Discosoma</i> sp. "red"
	<400>	27

cggtattgtg gccttcgta 19

35	<210>	28
	<211>	19

<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
5 <223> gene-specific primer used for 5'-RACE for
Discosoma striata
<400> 28
ttgtcttctt ctgcacaac 19

10 <210> 29
<211> 17
<212> DNA
<213> artificial sequence
<220>
15 <221> primer_bind
<223> gene-specific primer used for 5'-RACE for
Discosoma striata
<400> 29
ctgcacaacg ggtccat 17

20
<210> 30
<211> 20
<212> DNA
<213> artificial sequence
25 <220>
<221> primer_bind
<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata
<400> 30

30 cctctatctt catttctgc 20

<210> 31
<211> 20
<212> DNA
35 <213> artificial sequence
<220>
<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata

<400> 31

tatcttcatt tcctgcgtac 20

5

<210> 32

<211> 19

<212> DNA

<213> artificial sequence

10

<220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma sp. "magenta"

<400> 32

15 ttcagcaccc catcacgag 19

<210> 33

<211> 19

<212> DNA

20

<213> artificial sequence

<220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma sp. "magenta"

25

<400> 33

acgctcagag ctgggttcc 19

<210> 34

<211> 22

30

<212> DNA

<213> artificial sequence

<220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma sp. "green"

35

<400> 34

ccctcagcaa tccatcacgt tc 22

5 <210> 35
 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
 10 *Discosoma* sp. "green"
 <400> 35

attatctcag tggatgggttc 20

15 <210> 36
 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 20 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia majano*
 <400> 36

acatggatcc gctctttcaa acaagtttat c 31

25 <210> 37
 <211> 34
 <212> DNA
 <213> artificial sequence
 <220>
 30 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Anemonia majano*
 <400> 37

tagtactcga gcttattcgt atttcagtga aatc 34

35 <210> 38
 <211> 29

<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
5 <223> upstream primer used to obtain full coding region
of nFPs from *Clavularia sp.*
<400> 38
acatggatcc aacatttttt tgagaaacg 29
10 <210> 39
<211> 28
<212> DNA
<213> artificial sequence
<220>
15 <221> primer_bind
<223> upstream primer used to obtain full coding region
of nFPs from *Clavularia sp.*
<400> 39
acatggatcc aaagctctaa ccaccatg 28
20 <210> 40
<211> 31
<212> DNA
<213> artificial sequence
25 <220>
<221> primer_bind
<223> downstream primer used to obtain full coding
region of nFPs from *Clavularia sp.*
<400> 40
30 tagtactcga gcaacacaaa ccctcagaca a 31
<210> 41
<211> 28
<212> DNA
35 <213> artificial sequence
<220>

	<221>	primer_bind	
	<223>	upstream primer used to obtain full coding region of nFPs from <i>Zoanthus sp.</i>	
	<400>	41	
5	acatggatcc gctcagtc	aa agcacggt	28
	<210>	42	
	<211>	32	
	<212>	DNA	
10	<213>	artificial sequence	
	<220>		
	<221>	primer_bind	
	<223>	downstream primer used to obtain full coding region of nFPs from <i>Zoanthus sp.</i>	
15	<400>	42	
	tagtactcga ggttggaact	acattcttat ca	32
	<210>	43	
	<211>	31	
20	<212>	DNA	
	<213>	artificial sequence	
	<220>		
	<221>	primer_bind	
	<223>	upstream primer used to obtain full coding region of nFPs from <i>Discosoma sp.</i> "red"	
25	<400>	43	
	acatggatcc aggtcttcca	agaatgttat c	31
	<210>	44	
30	<211>	29	
	<212>	DNA	
	<213>	artificial sequence	
	<220>		
	<221>	primer_bind	
35	<223>	downstream primer used to obtain full coding region of nFPs from <i>Discosoma sp.</i> "red"	
	<400>	44	

tagtactcga ggagccaagt tcagcctta 29

5 <210> 45
 <211> 28
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 10 of nFPs from *Discosoma striata*
 <400> 45

acatggatcc agttggtcca agagtgtg 28

15 <210> 46
 <211> 28
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 20 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma striata*
 <400> 46

tagcgagctc tatcatgcct cgtcacct 28

25 <210> 47
 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 30 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia sulcata*
 <400> 47

acatggatcc gcttcctttt taaagaagac t 31

35 <210> 48
 <211> 28

<212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 5 <223> downstream primer used to obtain full coding
 region of nFPs from *Anemonia sulcata*
 <400> 48
 tagtactcga gtccttggga gcggttg 28
 10 <210> 49
 <211> 30
 <212> DNA
 <213> artificial sequence
 <220>
 15 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 of nFPs from *Discosoma* sp. "magenta"
 <400> 49
 acatggatcc agttgttcca agaattgat 30
 20 <210> 50
 <211> 26
 <212> DNA
 <213> artificial sequence
 25 <220>
 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma* sp. "magenta"
 <400> 50
 30 tagtactcga ggccattacg ctaatc 26
 <210> 51
 <211> 31
 <212> DNA
 35 <213> artificial sequence
 <220>
 <221> primer_bind

<223> upstream primer used to obtain full coding region
 of nFPs from *Discosoma sp.* "green"
 <400> 51
 acatggatcc agtgcactta aagaagaaat g 31
 5
 <210> 52
 <211> 29
 <212> DNA
 <213> artificial sequence
 10 <220>
 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma sp.* "green"
 <400> 52
 15 tagtactcga gattcggttt aatgccttg 29
 <210> 53
 <211> 33
 <212> DNA
 20 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> TS-oligo used in cDNA synthesis and RACE
 <400> 53
 25 aagcagtggg atcaacgcag agtacgcrgr grg 33
 <210> 54
 <211> 238
 <212> PRT
 30 <213> *Aequorea victoria*
 <220>
 <223> amino acid sequence of GFP
 <400> 54
 Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 35 5 10 15
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser

		20		25		30
	Gly Glu Gly Glu	Gly Asp Ala Thr Tyr	Gly Lys Leu Thr Leu Lys			
		35	40	45		
	Phe Ile Cys Thr	Thr Gly Lys Leu Pro Val	Pro Trp Pro Thr Leu			
5		50	55	60		
	Val Thr Thr Phe	Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro				
		65	70	75		
	Asp His Met Lys	Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu				
		80	85	90		
10	Gly Tyr Val Gln	Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn				
		95	100	105		
	Tyr Lys Thr Arg	Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val				
		110	115	120		
	Asn Arg Ile Glu	Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn				
15		125	130	135		
	Ile Leu Gly His	Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val				
		140	145	150		
	Tyr Ile Met Ala	Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe				
		155	160	165		
20	Lys Ile Arg His	Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp				
		170	175	180		
	His Tyr Gln Gln	Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu				
		185	190	195		
	Pro Asp Asn His	Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp				
25		200	205	210		
	Pro Asn Glu Lys	Arg Asp His Met Val Leu Leu Glu Phe Val Thr				
		215	220	225		
	Ala Ala Gly Ile	Thr His Gly Met Asp Glu Leu Tyr Lys				
		230	235			

30

<210> 55

<211> 862

<212> DNA

<213> *Anemonia majano*

35

<220>

<221> CDS

<223> cDNA sequence of wild type amFP486

<400> 55

```

gggagttcat tttggtcggc gacgtagtgg actacgaaaa ctcaactcga 50
ctttcgttca gttttgagaa acaagcgatt tgattcgaca tggctctttc 100
aaacaagttt atcggagatg acatgaaaat gacctaccat atggatggct 150
gtgtcaatgg gcattacttt accgtcaaag gtgaaggcaa cggaagcca 200
5  tacgaagggg cgcagacttc gactttttaa gtcaccatgg ccaacggtgg 250
gccctttgca ttctcctttg acatactatc tacagtgttc aaatatggaa 300
atcgatgctt tactgcgtat cctaccagta tgcccgacta tttcaaacia 350
gcatttcctg acggaatgtc atatgaaagg acttttacct atgaagatgg 400
aggagtgtgct acagccagtt gggaaataag ccttaaaggc aactgctttg 450
10 agcaciaaatc cacgtttcat ggagtgaact ttctgtctga tggacctgtg 500
atggcgaaaga agacaactgg ttggggaccca tcttttgaga aaatgactgt 550
ctgcatgaga atattgaagg gtgatgtcac cgcgttcctc atgctgcaag 600
gaggtggcaa ttacagatgc caattccaca cttcttacia gacaaaaaaaa 650
ccggtgacga tgccacaaa ccatgtggtg gaacatcgca ttgagaggac 700
15 cgaccttgac aaaggtggca acagtgttca gctgacggag cacgctgttg 750
cacatataac ctctgttgtc cctttctgag caaaaagttc gtttttagacc 800
ccgatttcac tgaaatacga ataagggttg cagaataata aagccgcaca 850
tttgaaataa tc 862

```

```

20      <210>      56
          <211>      229
          <212>      PRT
          <213>      Anemonia majano
          <220>
25      <223>      amino acid sequence of wild type amFP486
          <400>      56
Met Ala Leu Ser Asn Lys Phe Ile Gly Asp Asp Met Lys Met Thr
          5              10              15
Tyr His Met Asp Gly Cys Val Asn Gly His Tyr Phe Thr Val Lys
30          20              25              30
Gly Glu Gly Asn Gly Lys Pro Tyr Glu Gly Thr Gln Thr Ser Thr
          35              40              45
Phe Lys Val Thr Met Ala Asn Gly Gly Pro Leu Ala Phe Ser Phe
          50              55              60
35  Asp Ile Leu Ser Thr Val Phe Lys Tyr Gly Asn Arg Cys Phe Thr
          65              70              75
Ala Tyr Pro Thr Ser Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro

```

		80		85		90
	Asp Gly Met Ser Tyr Glu Arg Thr Phe Thr Tyr Glu Asp Gly Gly					
		95		100		105
	Val Ala Thr Ala Ser Trp Glu Ile Ser Leu Lys Gly Asn Cys Phe					
5		110		115		120
	Glu His Lys Ser Thr Phe His Gly Val Asn Phe Pro Ala Asp Gly					
		125		130		135
	Pro Val Met Ala Lys Lys Thr Thr Gly Trp Asp Pro Ser Phe Glu					
		140		145		150
10	Lys Met Thr Val Cys Asp Gly Ile Leu Lys Gly Asp Val Thr Ala					
		155		160		165
	Phe Leu Met Leu Gln Gly Gly Gly Asn Tyr Arg Cys Gln Phe His					
		170		175		180
	Thr Ser Tyr Lys Thr Lys Lys Pro Val Thr Met Pro Pro Asn His					
15		185		190		195
	Val Val Glu His Arg Ile Ala Arg Thr Asp Leu Asp Lys Gly Gly					
		200		205		210
	Asn Ser Val Gln Leu Thr Glu His Ala Val Ala His Ile Thr Ser					
		215		220		225
20	Val Val Pro Phe					

	<210>	57
	<211>	690
25	<212>	DNA
	<213>	artificial sequence
	<220>	
	<223>	nucleotide sequence of Mut15
	<400>	57
30	atggctcttt caaacaagtt tatcggagat gacatgaaaa tgacctacca	50
	tatggatggc tgtgtcaatg ggcattactt tacctgcaaa ggtgaaggca	100
	gcgggaagcc atacgaaggg acgcagacct cgacttttaa agtcaccatg	150
	gccaacggtg ggccccttgc attctccttt gacatactat ctacagtgtt	200
	cttgtatgga aatcgatgct ttactgcgta tcctaccagt atgcccgact	250
35	atttcaaaca agcatttcct gacggaatgt catatgaaag gacttttacc	300
	tatgaagatg gaggagttgc tacagccagt tgggaaataa gccttaaagg	350
	caactgcttt gagcacaaat ccacgtttca tggagtgaac tttcctgctg	400

atggacctgt gatggcgaag aagacaactg gttgggaccc atcttttgag 450
 aaaatgactg tctgcatgg aatattgaag ggtgatgtca ccgcgttcct 500
 catgctgcaa ggaggtggca attacagatg ccaattccac acttcctaca 550
 agacaaaaaa accggtgacg atgccaccaa accatgtggg ggaacatcgc 600
 5 attgcgagga ccgaccttga caaaggtggc aacagtgttc agctgacgga 650
 gcacgctgtt gcacatatataa cctctgttgt ccctttctga 690

<210> 58
 <211> 4695
 10 <212> DNA
 <213> artificial sequence
 <220>
 <223> nucleic acid sequence of vector pCNFPMut32-N1
 <400> 58

15 tagttattaa tagtaatcaa ttacgggggtc attagttcat agcccatata 50
 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg 100
 cccaacgacc cccgcccatt gacgtcaata atgacgtatg ttcccatagt 150
 aacgccaata gggactttcc attgacgtca atgggtggag tattttacggt 200
 aaactgcca cttggcagta catcaagtgt atcatatgcc aagtacgccc 250
 20 cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta 300
 catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 350
 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga 400
 tagcggtttg actcacgggg atttccaagt ctccacccca ttgacgtcaa 450
 tgggagtttg ttttggcacc aaaatcaacg ggactttcca aaatgtcgta 500
 25 acaactccgc ccattgacg caaatgggcg gtaggcgtgt acgggtgggag 550
 gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
 ccggactcag atctcgagct caagcttcga attctgcagt cgacgggtacc 650
 gcgggcccgg gatccggtac catggctctt tcaaacaagt ttatcggaga 700
 tgacatgaaa atgacctacc atatggatgg ctgtgtcaat gggcattact 750
 30 ttaccgtcaa aggtgaaggc aacgggaagc catacgaagg gacgcagact 800
 tcgactttta aagtcaccat ggccaacggg gggccccttg cattctcctt 850
 tgacatacta tctacagtgt tcaaatatgg aaatcgatgc tttactgcgt 900
 atcctaccag catgcccgcac tattttcaaac aagcatttcc tgacggaatg 950
 tcatatgaaa ggacttttac ctatgaagat ggaggagtgt ctacagccag 1000
 35 ttgggaaata agccttaaag gcaactgctt tgagcacaaa tccacgtttc 1050
 atggagtga ctttcctgct gatggacctg tgatggcgaa gaagacaact 1100
 ggttgggacc catcttttga gaaaatgact gtctgcgatg gaatattgaa 1150

gggatgatgtc accgcgttcc tcatgctgca aggaggtggc aattacagat 1200
 gccaatccca cacttcttac aagacaaaaa aaccggtgac gatgccacca 1250
 aaccatgtgg tggaacatcg cattgcgagg accgaccttg acaaaggtgg 1300
 caacagtgtt cagctgacgg agcacgctgt tgcacatata acctctgttg 1350
 5 tccctttctg agcgcccgcg actctagatc ataatacagcc ataccacatt 1400
 tgtagagggtt ttacttgctt taaaaaacct cccacacctc cccctgaacc 1450
 tgaaacataa aatgaatgca attgttggtt ttaacttggt tattgcagct 1500
 tataatgggtt acaataaag caatagcatc acaaatttca caaataaagc 1550
 attttttttca ctgcattcta gttgtggttt gtccaaactc atcaatgtat 1600
 10 cttaaggcgt aaattgtaag cgtaaatatt ttgttaaaat tcgcgttaaa 1650
 tttttgttaa atcagctcat tttttaacca ataggccgaa atcggcaaaa 1700
 tcccttataa atcaaaagaa tagaccgaga tagggttgag tgttgttcca 1750
 gtttggaaca agagtccact attaaagaac gtggactcca acgtcaaagg 1800
 gcgaaaaacc gtctatcagg gcgatggccc actacgtgaa ccatcaccct 1850
 15 aatcaagttt tttggggtcg aggtgccgta aagcactaaa tcggaaccct 1900
 aaaggaggcc cccgatttag agcttgacgg ggaaagccgg cgaacgtggc 1950
 gagaaaggaa gggaagaaag cgaaaggagc gggcgctagg gcgctggcaa 2000
 gtgtagcggc cacgctgcgc gtaaccacca caccgcgcgc gcttaatgcg 2050
 ccgctacagg gcgcgtcagg tggcactttt cggggaaatg tgcgcggaac 2100
 20 ccctatttgt ttatttttct aaatacatc aaatatgtat ccgctcatga 2150
 gacaataacc ctgataaatg cttcaataat attgaaaaag gaagagtcct 2200
 gaggcggaaa gaaccagctg tggaatgtgt gtcagttagg gtgtggaaaag 2250
 tccccaggct cccagcagg cagaagtatg caaagcatgc atctcaatta 2300
 gtcagcaacc aggtgtggaa agtccccagg ctccccagca ggcagaagta 2350
 25 tgcaaagcat gcatctcaat tagtcagcaa ccatagtccc gccctaact 2400
 ccgcccattc cgcccctaac tccgcccagt tccgcccatt ctccgcccga 2450
 tggctgacta atttttttta ttatgcaga ggccgaggcc gcctcggcct 2500
 ctgagctatt ccagaagtag tgaggaggct tttttggagg cctaggcttt 2550
 tgcaaagatc gatcaagaga caggatgagg atcgtttcgc atgattgaac 2600
 30 aagatggatt gcacgcagg tctccggccg cttgggtgga gaggctattc 2650
 ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtgtt 2700
 ccggctgtca gcgcaggggc gcccggttct ttttgtcaag accgacctgt 2750
 ccggtgccct gaatgaactg caagacgagg cagcgcggct atcgtggctg 2800
 gccacgacgg gcgttccttg cgcagctgtg ctgcacgttg tcaactgaagc 2850
 35 ggggaaggac tggctgctat tgggcgaagt gccggggcag gatctcctgt 2900
 catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 2950
 cggcggctgc atacgcttga tccggctacc tgcccattcg accaccaagc 3000
 gaaacatcgc atcgagcgag cacgtactcg gatggaagcc ggtcttgctg 3050

atcaggatga tctggacgaa gagcatcagg ggctcgcgcc agccgaactg 3100
 ttcgccaggc tcaaggcgag catgcccagc ggcgaggatc tcgtcgtgac 3150
 ccatggcgat gcctgcttgc cgaatatcat ggtggaaaat ggccgctttt 3200
 ctggattcat cgactgtggc cggctgggtg tggcggaccg ctatcaggac 3250
 5 atagcgttgg ctaccctga tattgctgaa gagcttggcg gcgaatgggc 3300
 tgaccgcttc ctctgtcttt acggtatcgc cgctcccgat tcgcagcgca 3350
 tcgccttcta tcgccttctt gacgagttct tctgagcggg actctggggg 3400
 tcgaaatgac cgaccaagcg acgccaacc tgccatcacg agatttcgat 3450
 tccaccgcg ccttctatga aagggtgggc ttcggaatcg ttttccggga 3500
 10 cgccggctgg atgacctcc agcgcgggga tctcatgctg gagttcttcg 3550
 cccaccctag ggggaggcta actgaaacac ggaaggagac aataccggaa 3600
 ggaaccgcg ctatgacggc aataaaaaga cagaataaaa cgcacggtgt 3650
 tgggtcgttt gttcataaac gcggggttcg gtcccagggc tggcactctg 3700
 tcgatacccc accgagacc cttggggcc aatacgcccg cgtttcttcc 3750
 15 ttttccccac cccaccccc aagtctgggt gaaggcccag ggctcgcacg 3800
 caacgtcggg gcggcaggcc ctgccatagc ctcaggttac tcatatatac 3850
 tttagattga tttaaaactt catttttaat ttaaaaggat ctaggtgaag 3900
 atcctttttg ataatctcat gacaaaaatc ccttaacgtg agttttcgtt 3950
 ccactgagcg tcagaccccg tagaaaagat caaaggatct tcttgagatc 4000
 20 ctttttttct gcgcgtaatc tgctgcttgc aaacaaaaaa accaccgcta 4050
 ccagcgggtg tttgtttgcc ggatcaagag ctaccaactc ttttccgaa 4100
 ggtaactggc ttcagcagag cgcagatacc aaatactgtc cttctagtgt 4150
 agccgtagtt aggccaccac ttcaagaact ctgtagcacc gcctacatac 4200
 ctcgctctgc taatcctgtt accagtggct gctgccagtg gcgataagtc 4250
 25 gtgtcttacc gggttggact caagacgata gttaccggat aaggcgcagc 4300
 ggtcgggctg aacggggggg tcgtgcacac agcccagctt ggagcgaacg 4350
 acctacaccg aactgagata cctacagcgt gagctatgag aaagcgccac 4400
 gcttcccga aaggagaaagg cggacaggta tccggtaagc ggcagggtcg 4450
 gaacaggaga gcgcacgagg gagcttccag ggggaaacgc ctggtatctt 4500
 30 tatagtcttg tcgggtttcg ccacctctga cttgagcgtc gattttttgtg 4550
 atgctcgtca ggggggcgga gcctatggaa aaacgccagc aacgcggcct 4600
 ttttacgggt cctggccttt tgctggcctt ttgctcacat gttctttcct 4650
 gcgttatccc ctgattctgt ggataaccgt attaccgcca tgcac 4695

35 <210> 59
 <211> 4821
 <212> DNA

<213> artificial sequence

<220>

<223> nucleic acid sequence of vector pCNFP-MODCd1

<400> 59

```

5  tagttattaa tagtaatcaa ttacgggggtc attagttcat agcccatata 50
   tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg 100
   cccaacgacc cccgcccatt gacgtcaata atgacgtatg ttcccatagt 150
   aacgccaata gggactttcc attgacgtca atgggtggag tatttacggg 200
   aaactgcca cttggcagta catcaagtgt atcatatgcc aagtacgccc 250
10 cctattgacg tcaatgacgg taaatggccc gcctggcatt atgccagta 300
   catgacctta tgggactttc ctacttggca gtacatctac gtattagtca 350
   tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga 400
   tagcggtttg actcacgggg atttccaagt ctccacccca ttgacgtcaa 450
   tgggagtttg ttttggcacc aaaatcaacg ggactttcca aaatgtcgta 500
15 acaactccgc ccattgacg caaatgggcg gtaggcgtgt acggtgggag 550
   gtctatataa gcagagctgg tttagtgaac cgtcagatcc gctagcgcta 600
   ccggactcag atctcgagct caagcttcga attctgcagt cgacggtacc 650
   gcgggcccgg gatccggtac catggctctt tcaaacaagt ttatcggaga 700
   tgacatgaaa atgacctacc atatggatgg ctgtgtcaat gggcattact 750
20 ttaccgtcaa aggtgaaggc aacgggaagc catacgaagg gacgcagact 800
   tcgactttta aagtcaccat ggccaacggt gggccccttg cattctcctt 850
   tgacatacta tctacagtgt tcaaatatgg aaatcgatgc tttactgctg 900
   atcctaccag catgcccagc tatttcaaac aagcatttcc tgacggaatg 950
   tcatatgaaa ggacttttac ctatgaagat ggaggagtgt ctacagccag 1000
25 ttgggaaata agccttaaag gcaactgctt tgagcacaaa tccacgtttc 1050
   atggagttaa ctttcctgct gatggacctg tgatggcgaa gaagacaact 1100
   ggttgggacc catcttttga gaaaatgact gtctgcgatg gaatattgaa 1150
   ggggtgatgtc accgcgttcc tcatgctgca aggaggtggc aattacagat 1200
   gccaatcca cacttcttac aagacaaaaa aaccggtgac gatgccacca 1250
30 aaccatgtgg tggaacatcg cattgcgagg accgacctg acaaagggtg 1300
   caacagtgtt cagctgacgg agcacgctgt tgcacatata acctctgttg 1350
   tccctttcaa gcttagccat ggcttccgc cggcggtggc ggcgcaggat 1400
   gatggcacgc tgcccatgtc ttgtgcccag gagagcggga tggaccgtca 1450
   ccctgcagcc tgtgcttctg ctaggatcaa tgtgtaggcg gccgcgactc 1500
35 tagatcataa tcagccatac cacatttgta gaggttttac ttgctttaa 1550
   aaacctcca cacctcccc tgaacctgaa acataaaatg aatgcaattg 1600
   ttgttgtaa cttgtttatt gcagcttata atggttacaa ataaagcaat 1650
   agcatcacia atttcacaaa taaagcattt ttttactgc attctagttg 1700

```

5 tggtttgtcc aaactcatca atgtatctta aggcgtaa at tgtaagcggt 1750
 aatattttgt taaaattcgc gttaaatttt tgttaaatca gctcattttt 1800
 taaccaatag gccgaaatcg gcaaaatccc ttataaatca aaagaataga 1850
 ccgagatagg gttgagtggt gttccagttt ggaacaagag tccactatta 1900
 aagaacgtgg actccaacgt caaagggcga aaaaccgtct atcagggcga 1950
 tggcccacta cgtgaaccat caccctaate aagttttttg gggtcgaggt 2000
 gccgtaaagc actaaatcgg aaccctaag ggagcccccg atttagagct 2050
 tgacggggaa agccggcgaa cgtggcgaga aaggaagga agaaagcgaa 2100
 aggagcgggc gctagggcgc tggcaagtgt agcggtcacg ctgcgcgtaa 2150
 10 ccaccacacc cgccgcgctt aatgcgccgc tacagggcgc gtcaggtggc 2200
 acttttcggg gaaatgtgcg cggaacccct atttgtttat ttttctaaat 2250
 acattcaa atgtatccgc tcatgagaca ataaccctga taaatgcttc 2300
 aataatattg aaaaaggaag agtcctgagg cggaagaac cagctgtgga 2350
 atgtgtgtca gttaggggtg ggaaagtccc caggctcccc agcaggcaga 2400
 15 agtatgcaaa gcatgcatct caattagtca gcaaccaggt gtggaaagtc 2450
 cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt 2500
 cagcaaccat agtcccgc ctaactccgc ccatcccgc cctaactccg 2550
 cccagttccg ccatttctcc gcccctggc tgactaattt tttttattta 2600
 tgcagaggcc gagggcgct cgccctctga gctattccag aagtagtgag 2650
 20 gaggcttttt tggaggccta ggcttttgca aagatcgatc aagagacagg 2700
 atgaggatcg tttcgcatga ttgaacaaga tggattgcac gcaggttctc 2750
 cggccgcttg ggtggagagg ctattcggct atgactgggc acaacagaca 2800
 atcggctgct ctgatgccgc cgtgttccgg ctgtcagcgc aggggcgccc 2850
 ggttcttttt gtcaagaccg acctgtccgg tgccctgaat gaactgcaag 2900
 25 acgaggcagc gcggctatcg tggctggcca cgacgggcgt tccttgcgca 2950
 gctgtgctcg acgttggtcac tgaagcggga agggactggc tgctattggg 3000
 cgaagtgcg gggcaggatc tcctgtcatc tcacctgct cctgccgaga 3050
 aagtatccat catggctgat gcaatgcggc ggctgcatac gcttgatccg 3100
 gctacctgcc cattcgacca ccaagcgaaa catcgcatcg agcagcacg 3150
 30 tactcggatg gaagccggtc ttgtcgatca ggatgatctg gacgaagagc 3200
 atcaggggct cgccgcagcc gaactgttcg ccaggctcaa ggcgagcatg 3250
 cccgacggcg aggatctcgt cgtgacccat ggcatgcct gcttgccgaa 3300
 tatcatggtg gaaaatggc gcttttctgg attcatcgac tgtggccggc 3350
 tgggtgtggc ggaccgctat caggacatag cgttggttac ccgtgatatt 3400
 35 gctgaagagc ttggcggcga atgggctgac cgcttcctcg tgctttacgg 3450
 tatcgccgct cccgattcgc agcgcacgc cttctatcgc cttcttgacg 3500
 agttcttctg agcgggactc tggggttcga aatgaccgac caagcgacgc 3550
 ccaacctgcc atcacgagat ttcgattcca ccgccgcctt ctatgaaagg 3600

```

ttgggcttcg gaatcgtttt cggggacgcc ggctggatga tcctccagcg 3650
cgggggatctc atgctggagt tcttcgcccc ccctaggggg aggctaactg 3700
aaacacggaa ggagacaata cgggaaggaa cccgcgctat gacggcaata 3750
aaaagacaga ataaaacgca cggtggttggg tcgtttgttc ataaacgcgg 3800
5  ggttcgggtcc cagggctggc actctgtcga taccacccg agacccatt 3850
ggggccaata cgcccgctt tcttcctttt cccacccca cccccaagt 3900
tcgggtgaag gcccgaggct cgcagccaac gtcggggcgg caggccctgc 3950
catagcctca ggttactcat atatacttta gattgattta aaacttcatt 4000
ttaatttaa aaggatctag gtgaagatcc tttttgataa tctcatgacc 4050
10  aaaatccctt aacgtgagtt ttcgttccac tgagcgtcag accccgtaga 4100
aaagatcaaa ggatcttctt gagatccttt ttttctgcgc gtaatctgct 4150
gcttgcaaac aaaaaaacca ccgctaccag cggtggtttg tttgccggat 4200
caagagctac caactctttt tccgaaggta actggcttca gcagagcgca 4350
gataccaaat actgtccttc tagtgtagcc gtagttaggc caccattca 4300
15  agaactctgt agcaccgcct acatacctcg ctctgctaata cctgttacca 4350
gtggctgctg ccagtggcga taagtcgtgt cttaccgggt tggactcaag 4400
acgatagtta ccggataagg cgcagcggtc gggctgaacg gggggttcgt 4450
gcacacagcc cagcttggag cgaacgacct acaccgaact gagataccta 4500
cagcgtgagc tatgagaaag cgccacgctt cccgaaggga gaaaggcgga 4550
20  caggtatccg gtaagcggca gggtcggaac aggagagcgc acgagggagc 4600
ttccaggggg aaacgcctgg tatctttata gtctgtcgg gtttcgccac 4650
ctctgacttg agcgtcgatt tttgtgatgc tcgtcagggg ggcgagcct 4700
atggaaaaac gccagcaacg cggccttttt acggttcctg gccttttgct 4750
ggccttttgc tcacatgttc tttcctgcgt tatccctga ttctgtggat 4800
25  aaccgtatta ccgcatgca t 4821

```

<210> 60

<211> 4621

<212> DNA

30 <213> artificial sequence

<220>

<223> nucleic acid sequence of vector pCNFP-MODCd2

<400> 60

```

tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata 50
35  tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg 100
cccaacgacc cccgccatt gacgtcaata atgacgtatg ttcccatagt 150
aacgccaata gggactttcc attgacgtca atgggtggag tatttacggt 200

```

	aaactgcccc	cttggcagta	catcaagtgt	atcatatgcc	aagtacgccc	250
	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt	atgcccagta	300
	catgacctta	tgggactttc	ctacttggca	gtacatctac	gtattagtca	350
	tcgctattac	catggtgatg	cggttttggc	agtacatcaa	tgggcgtgga	400
5	tagcgggtttg	actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	450
	tgggagtttg	ttttggcacc	aaaatcaacg	ggactttcca	aaatgtcgta	500
	acaactccgc	cccattgacg	caaatgggcg	gtaggcgtgt	acggtgggag	550
	gtctatataa	gcagagctgg	tttagtgaac	cgtcagatcc	gctagcgcta	600
	ccggactcag	atctcgagct	caagcttcga	attctgcagt	cgacgggtacc	650
10	gcgggccccg	gatccggtac	catggctctt	tcaaacaagt	ttatcggaga	700
	tgacatgaaa	atgacctacc	atatggatgg	ctgtgtcaat	gggcattact	750
	ttaccgtcaa	aggtgaaggc	aacgggaagc	catacgaagg	gacgcagact	800
	tcgactttta	aagtcaccat	ggccaacggt	gggccccttg	cattctcctt	850
	tgacatacta	tctacagtgt	tcaaatatgg	aaatcgatgc	tttactgcgt	900
15	atcctaccag	catgcccagc	tatttcaaac	aagcatttcc	tgacggaatg	950
	tcatatgaaa	ggacttttac	ctatgaagat	ggaggagttg	ctacagccag	1000
	ttgggaaata	agccttaaag	gcaactgctt	tgagcacaaa	tccacgtttc	1050
	atggagtga	ctttcctgct	gatggacctg	tgatggcgaa	gaagacaact	1100
	ggttgggacc	catcttttga	gaaaatgact	gtctgcgatg	gaatattgaa	1150
20	gggtgatgtc	accgcgttcc	tcagtctgca	aggaggtggc	aattacagat	1200
	gccaattcca	cacttcttac	aagacaaaaa	aaccggtgac	gatgccacca	1250
	aaccatgtgg	tggaaacatcg	cattgcgagg	accgaccttg	acaaagggtgg	1300
	caacagtgtt	cagctgacgg	agcacgctgt	tgcacatata	acctctgttg	1350
	tccctttcaa	gcttagccat	ggcttcccgc	cggaggtgga	ggagcaggat	1400
25	gatggcacgc	tgcccatgtc	ttgtgcccag	gagagcggga	tggaccgtca	1450
	ccctgcagcc	tgtgcttctg	ctaggatcaa	tgtgtaggcg	gccgcgactc	1500
	tagatcataa	tcagccatac	cacatttgta	gaggttttac	ttgcttttaa	1550
	aaacctcca	cacctcccc	tgaacctgaa	acataaaatg	aatgcaattg	1600
	ttgttggtta	cttgtttatt	gcagcttata	atggttacia	ataaagcaat	1650
30	agcatcacia	atttcacaaa	taaagcattt	ttttcactgc	attctagttg	1700
	tggtttgtcc	aaactcatca	atgtatctta	aggcgtaa	tgtaagcggt	1750
	aatattttgt	taaaattcgc	gttaaatttt	tgttaa	gctcattttt	1800
	taaccaatag	gccgaaatcg	gcaaaatccc	ttataaatca	aaagaataga	1850
	ccgagatagg	gttgagtgtt	gttccagttt	ggaacaagag	tccactatta	1900
35	aagaacgtgg	actccaacgt	caaagggcga	aaaaccgtct	atcagggcga	1950
	tggcccacta	cgtgaaccat	caccctaata	aagttttttg	gggtcgaggt	2000
	gccgtaaagc	actaaatcgg	aaccctaag	ggagcccccg	atttagagct	2050
	tgacggggaa	agccggcgaa	cgtggcgaga	aaggaaggga	agaaagcgaa	2100

aggagcgggc gctagggcgc tggcaagtgt agcgggtcacg ctgcgcgtaa 2150
 ccaccacacc cgccgcgctt aatgcgccgc tacagggcgc gtcaggtggc 2200
 acttttcggg gaaatgtgcg cggaaccctt atttgtttat ttttctaaat 2250
 acattcaa atgtatccgc tcatgagaca ataaccctga taaatgcttc 2300
 5 aataatattg aaaaaggaag agtcctgagg cggaagaac cagctgtgga 2350
 atgtgtgtca gttaggggtg ggaaagtccc caggctcccc agcaggcaga 2400
 agtatgcaaa gcatgcatct caattagtca gcaaccagggt gtggaaagtc 2450
 cccaggctcc ccagcaggca gaagtatgca aagcatgcat ctcaattagt 2500
 cagcaaccat agtcccgccc ctaactccgc ccatcccgcc cctaactccg 2550
 10 cccagttccg ccattctctc gcccctggc tgactaattt tttttattta 2600
 tgcagaggcc gagggccgct cgccctctga gctattccag aagtagtgag 2650
 gaggcttttt tggaggccta ggcttttgca aagatcgatc aagagacagg 2700
 atgaggatcg ttctgcatga ttgaacaaga tggattgcac gcaggttctc 2750
 cggccgcttg ggtggagagg ctattcgggt atgactgggc acaacagaca 2800
 15 atcggctgct ctgatgccgc cgtgttcggg ctgtcagcgc aggggcgccc 2850
 ggttcttttt gtcaagaccg acctgtccgg tgccctgaat gaactgcaag 2900
 acgaggcagc gcggctatcg tggctggcca cgacgggcgt tccttgcgca 2950
 gctgtgctcg acgttggtcac tgaagcggga agggactggc tgctattggg 3000
 cgaagtgcg gggcaggatc tcctgtcatc tcaccttgct cctgccgaga 3050
 20 aagtatccat catggctgat gcaatgcggc ggctgcatac gcttgatccg 3100
 gctacctgcc cattcgacca ccaagcga aa catcgcatcg agcgagcacg 3150
 tactcggatg gaagccggtc ttgtcgatca ggatgatctg gacgaagagc 3200
 atcaggggct cgcgccagcc gaactgttcg ccaggctcaa ggcgagcatg 3250
 cccgacggcg aggatctcgt cgtgacccat ggcgatgcct gcttgccgaa 3300
 25 tatcatggtg gaaaatggcc gcttttctgg attcatcgac tgtggccggc 3350
 tgggtgtggc ggaccgctat caggacatag cgttggctac ccgtgatatt 3400
 gctgaagagc ttggcggcga atgggctgac cgcttcctcg tgctttacgg 3450
 tatcgccgct cccgattcgc agcgcatcgc cttctatcgc cttcttgacg 3500
 agttcttctg agcgggactc tggggttcga aatgaccgac caagcgacgc 3550
 30 ccaacctgcc atcacgagat ttcgattcca ccgccgctt ctatgaaagg 3600
 ttgggcttcg gaatcgtttt ccgggacgcc ggctggatga tcctccagcg 3650
 cggggatctc atgctggagt tcttcgcca ccctaggggg aggctaactg 3700
 aaacacggaa ggagacaata ccggaaggaa cccgcgctat gacggcaata 3750
 aaaagacaga ataaaacgca cgggtgttggg tcgtttgttc ataaacgcgg 3800
 35 ggttcggtcc cagggtggtc actctgtcga taccaccacg agacccatt 3850
 ggggccaata cgcgcgctt tcttcctttt cccacccca cccccaagt 3900
 tcgggtgaag gcccgaggct cgcagccaac gtcggggcgg caggccctgc 3950
 catagcctca ggttactcat atatacttta gattgattta aaacttcatt 4000

```

      ttttaatttaa aaggatctag gtgaagatcc tttttgataa tctcatgacc 4050
      aaaatccctt aacgtgagtt ttcgttccac tgagcgtcag accccgtaga 4100
      aaagatcaaa ggatcttctt gagatccttt ttttctgcgc gtaatctgct 4150
      gcttgcaaac aaaaaaacca ccgctaccag cggtggtttg tttgccggat 4200
5     caagagctac caactctttt tccgaaggta actggcttca gcagagcgca 4250
      gataccaaat actgtccttc tagtgtagcc gtagttaggc caccacttca 4300
      agaactctgt agcaccgcct acatacctcg ctctgctaata cctgtttacca 4350
      gtggctgctg ccagtggcga taagtcgtgt cttaccgggt tggactcaag 4400
      acgatagtta ccggataagg cgcagcggtc gggctgaacg ggggggttcgt 4450
10    gcacacagcc cagcttggag cgaacgacct acaccgaact gagataccta 4500
      cagcgtgagc tatgagaaag cgccacgctt cccgaaggga gaaaggcgga 4550
      caggtatccg gtaagcggca gggtcggaac aggagagcgc acgagggagc 4400
      ttccaggggg aaacgcctgg tatctttata gtctgtcgg gtttcgccac 4450
      ctctgacttg agcgtcgatt tttgtgatgc tcgtcagggg ggcggagcct 4500
15    atggaaaaac gccagcaacg cggccttttt acggttcctg gccttttgct 4550
      ggccttttgc tcacatgttc tttcctgcgt tatccctga ttctgtggat 4600
      aaccgtatta ccgcatgca t                                     4621

```

```

      <210>      61
20    <211>      690
      <212>      DNA
      <213>      artificial sequence
      <220>
      <223>      nucleic acid sequence of humanized Mut32
25    <400>      61
      atggccctgt ccaacaagtt catcggcgac gacatgaaga tgacctacca 50
      catggacggc tgcgtgaacg gccactactt caccgtgaag ggcgagggca 100
      acggcaagcc ctacgagggc acccagacct ccaccttcaa ggtgaccatg 150
      gccaacggcg gccccctggc cttctccttc gacatcctgt ccaccgtgtt 200
30    caagtacggc aaccgctgct tcaccgccta cccaccagc atgcccgact 250
      acttcaagca ggccttcccc gacggcatgt cctacgagag aaccttcacc 300
      tacgaggacg gcggcgtggc caccgccagc tgggagatca gcctgaaggg 350
      caactgcttc gagcacaagt ccaccttcca cggcgtgaac ttccccgccg 400
      acggccccgt gatggccaag aagaccaccg gctgggaccc ctcttctcag 450
35    aagatgaccg tgtgcgacgg catcttgaag ggcgacgtga ccgccttcct 500
      gatgctgcag ggcgggcgga actacagatg ccagttccac acctcttaca 550
      agaccaagaa gcccgtgacc atgcccccca accacgtggt ggagcaccgc 600

```

atcgccagaa ccgacctgga caagggcggc aacagcgtgc agctgaccga 650
 gcacgccgtg gccacatca cctccgtggt gcccttctga 690

5 <210> 62
 <211> 26
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 10 <223> primer used to amplify human Marathon cDNA
 library (Burke's Lymphoma) to obtain mdm2 DNA
 <400> 62

atgtgcaata ccaacatgtc tgtacc 26

15 <210> 63
 <211> 21
 <212> DNA
 <213> artificial sequence
 <220>
 20 <221> primer_bind
 <223> primer used to amplify human Marathon cDNA
 library (Burke's Lymphoma) to obtain mdm2 DNA
 <400> 63

ctaggggaaa taagttagca c 21

25 <210> 64
 <211> 31
 <212> DNA
 <213> artificial sequence
 30 <220>
 <221> primer_bind
 <223> PCR primer used to add Kozac sequence and
 restriction sites
 <400> 64

35 ggaattccag ccatggtgtg caataccaac atgtctgtac c 31

<210> 65
<211> 26
<212> DNA
<213> artificial sequence
5 <220>
<221> primer_bind
<223> PCR primer used to add Kozac sequence and
restriction sites
<400> 65
10 tcccccgggg ggaaataagt tagcac 26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : (IPC 7): C07K 14/435; C12N 1/00, 1/15, 1/21, 5/10, 15/12, 15/63

US CL : Please See Extra Sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
****	The sequence diskette submitted with the description was defective; thus the documents listed below were obtained solely by a word search. No SEQ ID NOs. could be searched.	*****
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. October 1999. Nature Biotechnology, Volume 17, No. 10, pages 969-973, entire document.	1-34
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document	24-29, 30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 FEBRUARY 2000

Date of mailing of the international search report

17 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GABRIELE ELISABETH BUGAISKY

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANDERLUH et al. Cloning, sequencing and expression of equinatoxin II. 1996. Biochemical and Biophysical Research Communications. Volume 220, No. 2, pages 437-442, entire document.	1-5, 8, 12, 20-21, 23-28, 31
X	MACEK et al. Intrinsic tryptophan fluorescence of equinatoxin II, a pore-forming polypeptide from the sea anemone, Actinia equina	24-28, 31
A	L, monitors its interaction with lipid membranes. 1995. European Journal of Biochemistry, Volume 234, pages 329-335, entire document.	1-5, 8, 12, 20-21, 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29393

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

dialog files 155, 5, 434, 33, 357, 35 (Medline, Biosis, Scisearch, Oceanic Abs., Derwent Biotech. Abs., Dissertation Abs.); STN-CAS files Registry, CAPLUS; WEST files USPT, Derwent WPI

search terms: fluoresc? , Bioluminesc?, Protein, anthozo? , Zoanth?, Corallimorph?, Discosom?, Coral?, Alga, algae, discosom?, Cnidar?, Invert?, Rhodact?, Actinodisc?, Magenta, clavularia, zoanthus, anemonia, majano, anemon?, Zoanthar?, Actiniar?, Zoanthid?, Stolonif?, Alcyonar?, malsnkfig/sqsp, amfp486, striata, sulcata, Endomyar?